

K^+ CHANNELS IN XENOPUS SKELETAL MUSCLE

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K⁺ CHANNELS IN *XENOPUS* SKELETAL MUSCLE

by

William Mark Fry

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Abstract

The spatial distribution and identity of K^+ channels in embryonic *Xenopus* myocytes were investigated. Macropatch current recordings were used to determine the spatial distribution of inactivating (I_{IK}) and non-inactivating (I_K) outward K^+ currents as well as Na^+ currents (I_{Na}) in nerve-contacted *Xenopus* muscle cells grown in culture. Membrane patches either contained no detectable current or I_{Na} , and/or I_{IK} or I_K . More than 85% of the membrane patches which contained I_{Na} occurred within 40 μm of the nerve contact. In addition, more than 90% of the membrane patches which contained I_{Na} also contained I_{IK} . Sites of acetylcholine receptor localization at nerve-muscle contacts, identified by labeling with tetramethylrhodamine-labeled α -bungarotoxin, were also found to contain both I_{Na} (4/5 cells) and I_{IK} (5/5 cells). These results suggest that in *Xenopus* muscle cells in culture the channels mediating I_{IK} along with sodium channels are clustered at sites of synaptic specialization. To identify K^+ channels that underlie the observed K^+ currents in *Xenopus* muscle, a combination of RT-PCR, 5' RACE and 3' RACE was employed. Three K^+ channel cDNAs, designated XKv1.2', XKv1.4 and XKv1.10, were isolated. XKv1.2' and XKv1.4 cDNAs are *Xenopus* orthologues of known mammalian K^+ channels, however XKv1.10 is a novel Kv1 isoform. XKv1.4 mRNA appeared at stage 10.5 in whole embryos and was prominent in muscle throughout development from stage 14 to adult. XKv1.2' mRNA was detected by stage 11.5 in whole embryos, but remained at low levels in embryonic skeletal muscle (stages 14 and 21),

and was absent from adult muscle. XKv1.10 mRNA was first detected at stage 21 in whole embryos, and was present in muscle from this stage onwards. All three transcripts were present in spinal cord at stage 21. When transiently transfected into HEK 293 cells, XKv1.2' produced a non-inactivating K⁺ current similar to I_K, while XKv1.4 and XKv1.10 produced an inactivating K⁺ current similar to I_K. The results support the notion that the channel encoded by XKv1.2' may contribute to I_K, and channels encoded by XKv1.4 and XKv1.10 may contribute to I_K observed in embryonic muscle cells in culture. These K⁺ channels may be important in the development of skeletal muscle and the neuromuscular synapse.

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List of Abbreviations and symbols used

4-AP	4-aminopyridine
ACh	Acetylcholine
AChR	Acetylcholine receptor
ARIA	Acetylcholine receptor inducing activity; neuregulin
ATP	Adenosine 5' triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethanesulfonic acid
B _{KCa}	Large conductance Ca ⁺⁺ activated K ⁺ current
bp	base pairs
CaMKII	Ca ⁺⁺ /calmodulin dependent protein kinase II
cDNA	Complimentary deoxyribonucleic acid
cRNA	Complimentary ribonucleic acid
DEPC	Diethylpyrocarbonite
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECL	Entactin, collagen, laminin
EGF	Epidermal growth factor
EGFr	Epidermal growth factor receptor
E _K	Equilibrium potential for potassium
E _{Na}	Equilibrium potential for sodium
GDP	Guanosine 5' diphosphate
GFP	Green fluorescent protein
GTP	Guanosine 5' triphosphate
HEK	Human embryonic kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
I _A , I _K , I _{KA}	Inactivating voltage gated K ⁺ current
I _K , I _{Kv}	Non-inactivating voltage gated K ⁺ current
I _{KATP}	ATP gated K ⁺ current
I _{KNa}	Na ⁺ activated K ⁺ current
I _o	Low density, non-inactivating voltage gated K ⁺ current (<i>Xenopus</i> skeletal muscle)
I _{Na}	Voltage gated Na ⁺ current
MAGUK	Membrane associated guanylate kinase
MAP	Mitogen activated protein
MASC	Muscle associated specificity component
MLLV	Moloney murine leukemia virus
mRNA	Messenger ribonucleic acid
MuSK	Muscle specific kinase
NFM	Medium weight neurofilament
NMJ	Neuromuscular junction
nNOS	neuronal nitric oxide synthase
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC	Protein kinase C

RACE	Rapid amplification of cDNA ends
RATL	Rapsyn associated transmembrane linker
RMP	Resting membrane potential
RT	Reverse transcription/reverse transcriptase
S_{KCa}	Small conductance Ca^{++} activated K^{+} current
Sos	Son of sevenless
τ	Tau, time constant of current inactivation (ms)
TEA	Tetraethylammonium
Tris	tris(hydroxymethyl)aminomethane
TTX	Tetrodotoxin
TyrK	Tyrosine kinase
UTR	Untranslated region

Chapter 1

Introduction

1.1 Preamble

Our ability to run and jump, perceive our environment, or process language depends on the rapid electrical activity of neurons. The waves of electrical potential which are rapidly conducted along the length of a neuronal axon, and across the membrane of some muscle and glandular cells are called action potentials. The key to understanding action potentials or electrical excitability of cells is understanding "membrane theory". Membrane theory posits that the potential difference across a cell membrane is due to the differential distribution and concentration of charged ions across a selectively permeable membrane, and that rapid changes in membrane potential are due to changes in the selective permeability of the membrane. This thesis is about voltage gated K^+ channels, a type of protein ion channel that selectively allows the passage of K^+ across the cell membrane. The studies described herein aim to (1) characterize the spatial distribution and electrical properties of K^+ channels in the membrane of embryonic muscle cells with respect to sites of nerve contact (2) isolate cDNA clones encoding K^+ channels in embryonic skeletal muscle that may underlie the observed currents, (3) express the K^+ channel cDNA clones in a heterologous expression and characterize the K^+ currents they produce and (4) examine expression of the channel genes in developing embryos. But first I'll take a brief diversion into the history of

membrane theory, and the experiments that showed that ion channels are in fact true channels. This sets the stage to talk about the specific molecular structure of Na⁺ and K⁺ channels, the action potentials in skeletal muscle and the channels that underlie the ionic currents. Then I'll discuss what has been learned about the role of specific K⁺ channels during the development of electrical excitability in *Xenopus* muscle and spinal cord.

1.2 Membrane Theory

1.2.1 Historical perspective

Physiologists have known for a long time that ions were essential for electrical activity, and longer still that some form of electricity was essential for neuronal and muscular function. Luigi Galvani published a book in 1791, *De Vinbus Electricitatis in Mota Muscularis*, in which he proposed that muscle activity was based on animal electricity, and he stated that animal electricity and natural electricity were similar (Stevens, 1971). In the book he describes experiments where frog muscle was caused to contract in unison with the sparking activity of an electrical interference machine (static electricity generator), and also contract with atmospheric electricity. He compared muscle to a Leyden jar, and proposed that nerves, which are hollow and oily, conduct electric signals to muscle. Although he later erroneously claimed that nerve

conducted signals to muscle by movement of the electric fluid from the brain to muscle, his description of nerve was insightful in that he likened nerve conduction to that of an insulated wire which is an analogy commonly used today (Stevens, 1971).

The invention of the galvanometer in 1820 by Hans Christian Oersted during a physics lecture led science in new directions: a theory describing a link between electricity and magnetism was emerging, as was a theory proposing that the functioning of the nervous system involved some form of electricity (Clarke and O'Malley, 1968). Using a modified galvanometer, in 1827 Leopoldo Nobili detected the presence of an electric current in the decapitated frog. This was the first proof of animal electricity that could be measured with an instrument. Today we know that the current Nobili measured was related to the injury of the frog (muscle), and is partially due to loss of intracellular K^+ (Clarke and O'Malley, 1968). In 1841, Carlos Matteuchi was the first to show that contraction of an isolated muscle was accompanied by an electrical current. This current could stimulate an exposed motoneuron and subsequently cause the contraction of the muscle innervated by that motoneuron (Clarke and Jacyna, 1987). In 1844 A. C. Becquerel correctly interpreted this observation by proposing that the current produced by the first muscle during the contraction stimulated the motoneuron, as would a static electrical shock (Clarke and Jacyna, 1987).

Emil du Bois Reymond reconfirmed many of the observations made by Matteucci, including identification of a current associated with muscle contraction, and he coined this current the "action current" in 1843 (Clarke and O'Malley, 1968). Du Bois Reymond was a renowned master of instrumentation, and is credited as being the first to observe the nerve impulse, which he called a negative current variation (Clarke and Jacyna, 1987). He also made the insightful (but incorrect) proposal that currents observed in muscle and nerve were due to re-orientation of essentially non-mobile dipolar molecules inside tissues.

A few years later in 1850 a colleague of du Bois Reymond, Hermann Helmholtz, determined the velocity of electrical nerve impulses to be about 35 m/s. This value was surprising given that many scientists had expected the velocity of nerve impulses to be similar to the extremely high velocities of electric signals through telegraph wires (Stevens, 1971; Brazier, 1988). By now it was obvious that electricity was important to the physiology of muscle and nerve, but physiologists were still asking how did the electricity in muscle and nerve work?

In 1871 Julius Bernstein proposed the modern theory that the inactive nerve or muscle fiber is normally electrically polarized, with the external surface positive in relation to the internal one, and that the "action potential" (a word he coined) was a self-propagated depolarization of the membrane (Clarke and O'Malley, 1968). In the same year, H.P. Bowditch determined that the heart

muscle action potential was an all-or-nothing phenomenon, meaning that an electric shock would either induce a contraction, or fail to do so: the magnitude of the force of a single muscle contraction once elicited however was identical no matter how large the shock (Clarke and O'Malley, 1968). In 1914 Lord Edgar Douglass Adrian provided conclusive evidence of the presence of this all-or-nothing principle in nerve. He was also the first to show that sensory information from muscle back to the central nervous system (CNS) consisted of a series of action potentials where the frequency of action potentials was proportional to the intensity of the stimulus (Clarke and O'Malley, 1968).

Electrophysiologists often aimed to describe electrical phenomena in muscle and nerve in terms of simple circuits. In 1872 Ludimar Hermann realized that the travel of an action potential down the length of an axon was not down the core as water through a pipe, but was in fact the result of a number of small circuits out through the membrane, into the extracellular space, and back into the membrane (Hodgkin later renamed these as local circuits; Hille, 1992). Hermann also suggested that propagation of an action potential is a series of self stimulatory events and described the spread of potentials as though conducted along a "leaky telegraph cable", a proposal now known as the cable theory. (Hille, 1992).

In a series of landmark papers in the 1880s Ringer showed that a living frog heart must be bathed in a solution containing sodium, potassium and

calcium in definite proportions in order for the heart to keep beating (Hille, 1992). The significance of this may not be realized until one remembers that it was not until 1887 that Arrhenius showed that salts in solution dissociate into independent charged ions.

From 1888 to 1892, Nernst and Planck studied the thermodynamics of ionic diffusion potentials . The fruit of this work was the Nernst equation,

$$E_s = \frac{RT}{ZF} \ln \frac{[S_2]}{[S_1]}$$

which predicted that a potential difference E_s existed between two solutions, separated by a *selectively permeable membrane*, and could be calculated from the known concentrations of the ion $[S_2]$, and $[S_1]$ (Hille, 1992) . The equation was derived from a Boltzman equation which describes the relative probability of finding a particle in state 1 or state 2 given an energy difference between the two states. The RT/ZF were constants where R is the gas constant, T is the temperature in degrees Kelvin and F is Faraday's constant. Z was the valence of the ion

In 1890 Ostwald surmised that potentials of muscle nerve and the electric plaques of electric fish could be explained by asymmetric ion distributions and semipermeable membranes (Hille, 1967), but Bernstein was the first to apply the

Nernst equation to the living cell. Until 1902 there was little understanding of the relationship between resting potential and action current. Bernstein coined the term "Membrane Theory" in his attempt to synthesize a complete theory accounting for bioelectricity in terms of charged ions moving across a semipermeable membrane. He postulated that at rest, the membrane potential was due to selective diffusion of K^+ across the membrane. When the membrane became active, the selectivity for K^+ was lost and a generalized membrane breakdown temporarily allowed permeation of all ions. Erroneously, he thought the resting potential disappeared as excess negative ions inside the cell joined with excess positive ions from the outside. In spite of this error, he was the first to propose that permeation of ions across the membrane was responsible for the electrical properties of muscle and axon membrane (Brazier, 1988; Hille, 1992; Clarke and O'Malley, 1968; Cole, 1968).

For some years, Bernstein's theories remained largely untested because electrophysiologists at the time did not have the tools to directly measure a potential difference between the inside and outside of a sufficiently healthy cell, nor did they have the tools to measure ionic currents. However, in the summer of 1939 an American group of Kenneth Cole and Howard Curtis and a British group of Alan Lloyd Hodgkin, and A. F. Huxley were using more sophisticated electronics to study nerve impulses in the squid giant axon. Both groups had used glass micropipette electrodes inserted into squid axons, and measured a

resting potential difference of about 50 mV (the inside of the axon was -50 mV with respect to the outside of the cell) (Hodgkin and Huxley, 1939; Cole and Curtis, 1939). Cole and Curtis (Cole and Curtis, 1939; Curtis and Cole, 1940) also showed that during an action potential, the conductance of the squid giant axon increased by some 40-fold. This was strong evidence for Bernstein's theory as it showed that electrical activity took place across the cell membrane, and that the action potential was due to a transient increase in ionic conductance across the membrane. Curtis and Cole, (1942) also demonstrated that K^+ was the major ion determining resting potential when they added K^+ to the extracellular solution, and noted that the membrane potential dropped to zero. However some data collected from the squid axon conflicted with Bernstein's theory of membrane breakdown: Hodgkin and Huxley (1939; 1945) and Curtis and Cole (1940;1942) observed that the axonal membrane potential during an action potential overshoot zero mV by some tens of mV. This was not artefact and was not predicted by re-equilibrium of internal and external K^+ .

Hodgkin and Katz, (1949) rediscovered the requirement for the Na^+ for excitability: ironically, years earlier, in 1902, Overton wrote that Na^+ or Li^+ was essential for muscle contraction, and that movement of Na^+ or Li^+ across the membrane could carry an action current (Hille, 1992). This current could not be directly measured until 1947 when Cole's colleague Marmot invented the voltage clamp (Marmont, 1949). He threaded a thin wire down the length of a squid

axon and used a feedback amplifier to hold the entire axon at one potential (this technology was adapted from radar instrumentation developed during the war). The ability to record ionic currents at a given membrane potential was a major breakthrough: by "clamping" the membrane potential of the squid axon at various potentials and substituting ions in the extracellular solutions (Hodgkin *et al.*, 1952; Hodgkin and Huxley, 1952a,b,c,d) the group was able to quantify and mathematically model the permeation of Na^+ and K^+ across the axonal membrane. They demonstrated that the depolarization of membrane potential was carried by Na^+ entering the cell, and the repolarization was carried by K^+ leaving the cell. They demonstrated that the process of controlling (opening and closing) Na^+ and K^+ permeabilities called "gating", was highly complex and dependent on both time and membrane potential.

The development of the Hodgkin-Huxley model was perhaps one of the most important milestones in the development of "membrane theory" and physiology in general because it showed that K^+ and Na^+ traverse the membrane via distinct and independent pathways, each having separate voltage and time dependent kinetics. The Hodgkin-Huxley model explained the properties of the action potential and its propagation across a membrane, and put forth an explanation of membrane potential in terms of K^+ and Na^+ permeability. Hodgkin's group correctly postulated that the action potential is initiated when a small stimulus (electrical shock) depolarized the membrane

enough to open Na^+ pathways. The electrochemical gradient of Na^+ outside the cell favored its movement into the cell, so when the channels opened, Na^+ rushed down its electrochemical gradient, carried positive charges into the cell, and thus depolarized the membrane. The large membrane depolarization subsequently stimulated the Na^+ channels to close. Meanwhile, K^+ channels sensed the large depolarization of the cell membrane, and opened in response. Because the electrochemical gradient of K^+ favored its movement out of the cell, when the pathways opened K^+ flowed down its electrochemical gradient and out of the cell. This exiting of positive charges repolarized the membrane potential of the cell back towards resting potential. Thus the basis of the squid axon action potential was the specific gating of two separate ion permeation pathways. Once an understanding of the ionic basis of the action potential was accomplished, the question became "How do ions cross the membrane?"

1.2.2 Ion channels are distinct molecules that allow permeation across cell membranes

Until the 1960s, the permeation pathway of ions across the cell membrane was poorly understood. Experiments by Oersted in 1899 showed that highly charged species are not membrane permeant, so there had to be a dedicated pathway across which ions could move (Hille, 1992). The word "channel" was used to describe these pathways almost as soon as it was realized that the Na^+ and K^+ permeation pathways were in fact distinct (Hodgkin

et al., 1952). There were suggestions of spontaneously forming "holes" in the membrane, charged lipid-lined pathways, dedicated carriers, and water filled pores (Hille, 1992). The former two mechanisms were deemed thermodynamically unfavorable, but do appear in the literature. The latter two, the more plausible mechanisms, would require some kind of specialized molecule or structure. Pharmacological methods demonstrated that permeation pathways were distinct molecules. In 1964 Japanese scientists (Narahashi *et al.*, 1964) demonstrated that tetrodotoxin (TTX), the paralytic poison from pufferfish, selectively blocked Na^+ current in lobster giant axons and left K^+ current untouched. These results were soon confirmed in squid and frog axons (Hille, 1966; 1967; 1968). The sodium current block required only nM concentrations for complete block, was highly selective, and reversible. Another pharmacological agent, tetraethylammonium (TEA) was used in mM concentrations to block the K^+ currents in mollusc ganglion cells, squid axons, and nodes of Ranvier (Hagiwara and Saito, 1959). The importance of the pharmacological experiments was they showed that blocking ion currents required specific interaction of a toxin with a receptor (pharmacological receptor, as opposed to a neurotransmitter receptor). The most likely candidate for these receptors were the channels themselves, suggesting that the permeation pathways were individual molecules.

Two lines of biophysical evidence have pointed to water filled pores

acting as ion selective diffusion holes as opposed to transporter molecules. (1) Examination of single channels by noise analysis and more recently single channel recording showed that 0.6×10^7 to 12×10^7 ions per second may pass through the conduction pathway, but the maximal theoretical limit for a transporter to shuttle ions across the membrane is 10^5 ions per second (Miller, 1987). Furthermore, acetylcholinesterase, and carbonic anhydrase, enzymes with the fastest kinetic activities known, have maximal turnover rates of 10^5 molecules per second, and the fastest rates observed for ion transporters are 100 ions per second in the case of Na^+ and K^+ ATPase and Ca^{++} ATPase, and 1000 ions per second in the case of the erythrocyte chloride transporter (Miller, 1987); (2) Ion channels have low temperature coefficients (Hille, 1992) which implies that ion channels do not undergo large changes in conformation such as would be necessary to bind and shuttle an ion across the lipid bilayer.

By the 1970s the nicotinic acetylcholine receptor (AChR) channel found at muscle endplates and the voltage gated Na^+ channel had been identified as proteins. The AChR was biochemically purified from membrane fractions of the electric organs of the electric ray *Torpedo* using detergent extraction and alpha bungarotoxin (a specific AChR toxin) affinity columns (Weill *et al.*, 1974). The Na^+ channel was purified from electric eel using radioactive TTX: fractions of membrane containing the TTX bound sodium channel could be identified by their radioactivity (Agnew *et al.*, 1978). After partially sequencing these proteins,

molecular biological techniques were used to isolate complimentary DNA (cDNA) clones for the entire coding regions of the genes responsible for these proteins (Noda *et al.*, 1982; Numa *et al.*, 1983; Noda *et al.*, 1984).

Isolation of K⁺ channel protein and cDNA was not as straightforward as that of the AChR and Na⁺ channel. Electrophysiologists were able to record large K⁺ currents, but membrane biologists were unable to isolate K⁺ channel proteins. In contrast to AChRs and Na⁺ channels, there was no membrane that was a rich source from which to isolate K⁺ channel proteins (Rehm and Lazdunski, 1988; Schmidt and Betz, 1988). However, it was observed that *Shaker* mutants of *Drosophila* shook their legs under ether anaesthesia, and larvae showed an unusually prolonged release of neurotransmitter at the neuromuscular junction (NMJ). It was proposed that defective K⁺ channels found in nerve terminals may be involved with this phenomenon (Jan *et al.*, 1977; Salkoff and Wyman, 1981). Voltage clamp experiments showed that one of the voltage gated K⁺ currents, the inactivating current called I_A, was indeed abolished in the *Shaker* mutants (Salkoff, 1983). Soon after, a heroic combination of genetics and chromosome walking in *Drosophila* allowed the cloning of the first K⁺ channels from the *Shaker* locus of *Drosophila*.

The first fragment of the *Shaker* gene was isolated by Kamb *et al.*, (1987), but this group had identified only a portion of the *Shaker* cDNA encoding three putative membrane spanning domains. Soon after, others cloned a longer

segment of the *Shaker* cDNA, and this fragment encoded what appeared to be six membrane spanning regions (Tempel *et al.*, 1987). This cDNA showed immediate similarity to the S4 region of eel sodium Na⁺ channel previously cloned by Noda *et al.* (1984). The S4 region is characterized by repeated positively charged arginine residues, and was thus proposed to be the voltage sensor of the Na⁺ channel (Greenblatt *et al.*, 1985). Hydrophobicity analysis and comparison to the predicted structure for voltage gated Na⁺ channels predicted the *Shaker* protein had six hydrophobic membrane spanning regions S1-S6, and a shorter hydrophobic region which dipped into the membrane. Further analysis showed that the *Shaker* gene gave rise to several distinct products via gene splicing (Tempel *et al.*, 1987; Pongs *et al.*, 1988; Kamb *et al.*, 1987), and these had different spatial and temporal patterns of expression, and distinguishable kinetics. When expressed in *Xenopus* embryos, most *Drosophila Shaker* K⁺ channels produced an inactivating K⁺ current, and were rapidly blocked by 4-aminopyridine (4-AP) as were the native currents.

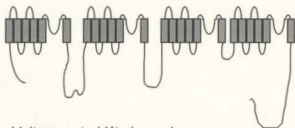
The K⁺ channels encoded by *Shaker* were similar to voltage gated Na⁺ and Ca²⁺ channels which had been previously cloned (Noda *et al.*, 1984; Tanabe *et al.*, 1987), but were much smaller: the molecular weight of predicted *Shaker* K⁺ channels was about 70 kd, but the Na⁺ channel was about 260 kd. This troubling fact was quickly addressed when the predicted membrane topology is examined. The *Shaker* K⁺ channel appeared to be equivalent to one of the four

domains present in the Na⁺ channel. The functional *Shaker* K⁺ channel was thus predicted to be a tetramer consisting of four subunits (Figure 1.1). This theory was supported by a number of studies where cDNAs of different alternatively spliced isoforms of *Shaker*, having different kinetic properties, were co-expressed in *Xenopus* oocytes (Christie *et al.*, 1989; MacKinnon, 1991; Timpe *et al.*, 1988; Ruppersberg *et al.*, 1990). The resulting K⁺ currents exhibited properties that were not simple mixtures of the two current types. The currents had properties intermediate to both. Furthermore, Isacoff *et al.*, (1990) constructed a clone of two contiguous *Shaker* cDNAs which produced functional channels when expressed in *Xenopus* oocytes. Thus the number of subunits in a functional K⁺ channel was even, supporting the notion that K⁺ channels are tetrameric structures.

The isolation of *Shaker* K⁺ channel led to an explosion in the cloning of many other K⁺ channel genes. Based on cDNA screening in *Drosophila* with *Shaker* specific probes, four groups of voltage gated K⁺ channels have been identified. These are (in addition to *Shaker*) *Shal*, *Shab* and *Shaw* (Wei *et al.*, 1990). The amino acid sequences of these K⁺ channels were highly conserved, about 40% between any two. Moreover, a multitude of K⁺ channel orthologues of these four groups has been isolated from a wide variety of organisms, from vertebrates and invertebrates alike. A *Shaker* orthologue has even been isolated from the one of the most ancient extant metazoans, the jellyfish (Jegla

Figure 1.1 Structure of voltage gated Na⁺ and K⁺ channels. Na⁺ channel proteins consist of four domains (I, II, III, IV), each containing six α -helical membrane spanning regions (S1-S6) and a pore region (P). The four domains fold to form a central water filled pore. K⁺ channels proteins consist of a single domain containing six α -helical membrane spanning domains (S1-S6) and a pore domain (P). Four such subunits assemble to form the functional K⁺ channel. The S4 regions of both channel types contain multiple positively charged amino acids and determine voltage sensitivity. The N-terminal of the K⁺ channel Kv1.4 isoform contains a "ball and chain" domain which can block the pore, thus causing inactivation (redrawn from Catterall, 1992; Jan and Jan, 1992)

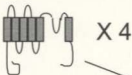
Voltage gated Na⁺ channel



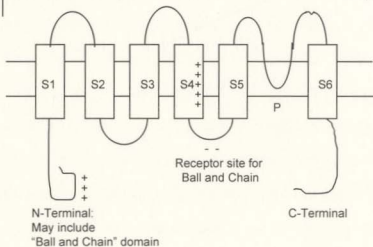
Fold



Voltage gated K⁺ channel



Fold and
assemble



et al., 1995). It has become clear that further diversity of the voltage gated K^+ channels within vertebrates is generated by the fact that within each group of K^+ channels (*Shaker*, *Shab*, etc) there exist numerous isoforms. With the rapid increase in number of K^+ channel clones the nomenclature of the voltage gated K^+ channels has been standardized. Each of the *Shaker*, *Shab*, *Shal*, and *Shaw* groups are considered to be subfamilies of the voltage gated K^+ channel family. The nomenclature reflects the hierarchy of this: *Shaker* is the Kv1 subfamily (Kv for voltage gated K^+ channel), *Shab* is Kv2, *Shaw* is Kv3 and *Shal* is Kv4. Isoforms of these are indicated by a decimal. For example Kv1.1, Kv1.2 and so on are isoforms within the Kv1 subfamily (Chandy and Gutman, 1993).

Although the specific subunit structure of Na^+ and K^+ channels as well as other channels such as AChRs and connexins that form gap junctions are quite different, the theme of ion channel structure remains the same. Multiple similar subunits or functional domains, which are membrane spanning proteins, associate in a symmetric fashion around a central, water filled pore. The ion channels may be closed or open, but when they receive the proper stimulus, such as membrane depolarization, or ligand binding the channels change configuration and ions are conducted across the membrane through the pore to move down their electrochemical gradient (or conversely close and stop ionic flow).

1.3 Na^+ and K^+ currents in skeletal muscle

1.3.1 Diversity

With the advent of the voltage clamp in the 1950s, many different tissues soon found their way under the microscopes and electrodes of the electrophysiologist. Several facts became clear. (1) The electrophysiological properties of large myelinated axons from vertebrates, arthropods, molluscs and annelids were all very similar (Hille, 1992). Action potentials were conducted in saltatory fashion down the length of a myelinated axon and most of the ionic conductance occurred at the nodes. The Na^+ current activated and inactivated rapidly and the K^+ current activated with a slight delay compared to the Na^+ current. The Na^+ and K^+ current kinetics could be fit to the Hodgkin-Huxley kinetic model with but minor variations. In addition, the pharmacological response of most axons to the drugs TTX and TEA were similar. This conservation of function reflected the fact that the only role of axons was to transmit electrical messages over distances. (2) The action potential of skeletal muscle was similar to that of the axon. Subthreshold stimuli produced similar passive responses across the membrane in both muscle and axon, and suprathreshold stimuli trigger characteristic all-or-nothing action potential whose propagation depends on local circuit current flow between excited and resting areas of membrane. The Na^+ and K^+ currents that were found to underlie the

action potential in frog sartorius muscle could also be fit to the Hodgkin-Huxley equations without much alteration (Adrian *et al.*, 1970b). However the role of action potentials in skeletal muscle is not simply to be transmitted from one end of a cell to the other, but instead is only one event in a sequence of electrical events that activate the contractile machinery of the muscle cell.

Most of the early voltage clamp experiments were carried out with the intent of comparing K^+ and Na^+ current activation and inactivation kinetics to Hodgkin-Huxley model. However Na^+ and K^+ currents in skeletal muscle were soon found to be more complex than originally thought, although they still could fit the Hodgkin-Huxley model with minor modifications. For example, (Redfern and Thesleff, 1971a,b) noted that action potentials in denervated mammalian skeletal muscle were relatively resistant to block by TTX: denervated muscle required μM concentrations to achieve block whereas innervated muscle required only nM concentrations. Action potentials in immature mammalian skeletal muscles were also shown to be resistant to TTX (Frelin *et al.*, 1983). Two Na^+ channel isoforms were shown to be differentially expressed in muscle, one of which was sensitive to TTX and the other was not (Trimmer *et al.*, 1989; Kallen *et al.*, 1990). Regulation of the expression of these two channels was under neural control (Sherman and Catterall, 1982; Yang *et al.*, 1991).

The K^+ current was often viewed only as the current that repolarized the cell after the membrane became depolarized, and thus it was seldom examined

after 5 ms. In 1970, a new type of K^+ current was observed in neurons (Connor and Stevens, 1971). This K^+ current showed rapid inactivation with maintained depolarization (inactivation time constant of current decay τ , of 10 ms), and was active at sub-threshold potentials. It was called A-current or I_A to distinguish it from the so-called delayed rectifier (hereinafter called non-inactivating K^+ current) present in the squid axon that showed no or very slow inactivation with maintained depolarization and activated slightly above the action potential threshold. The A-current thus had the unique property of regulating the frequency of action potentials. Interest in the roles of K^+ channels increased, and researchers began to look at the properties of K^+ currents in greater detail.

The time courses of K^+ currents in skeletal muscle were also found to vary. In mature amphibian skeletal muscle, Adrian *et al.*, (1970a,b) found that the outward K^+ current inactivated with a time constant of 400-600 ms at potentials near 0 mV, but interpretation was made difficult by the presence of two other K^+ currents, one of which was the inward rectifier (as described by Katz, 1949; Adrian and Freygang, 1962), and the other of which was very slowly activating (reached maximum current at about 3 s after depolarization). Two classes of voltage activated K^+ current have subsequently been described in mouse skeletal muscle. One class shows rapid inactivation, and is sensitive to aminopyridines (Duval and Leoty, 1978; Duval and Leoty, 1980a,b). The other class shows slower inactivation with a maintained depolarization, and shows

higher sensitivity to TEA (Duval and Leoty, 1980a; Duval and Leoty, 1980b; Stanfield, 1983). The fast inactivating component had a time constant τ of about 150-300 ms, while the slow component had a τ value of 1500-3000 ms (Duval and Leoty 1978;1980a;b). More recently, the voltage activated K^+ currents have been examined in skeletal muscle using whole cell and single channel patch clamp recording. In mouse interosseus muscle, the K^+ current was observed to have fast and slowly inactivating components. The fast inactivating component had a τ value of about 150 ms, while the slowly inactivating component had a τ value of 400 ms (Brinkmeier *et al.*, 1991). Two components of the K^+ current were also identified in mouse extensor digitorum longus muscle (Hocherman and Bezanilla, 1996). The fast component had a τ value on the order of 60-80 ms, while slowly inactivating component had a τ value on the order of 500-800 ms.

These results were obtained from acutely dissociated muscle cells which may be subject to membrane damage during isolation, but similar results have been observed in muscle cells growing in culture. Three voltage gated K^+ currents have been identified in embryonic *Xenopus* muscle in culture. One current, I_K , activated with depolarization, and did not significantly inactivate over 100 ms (Ribera and Spitzer, 1990; Moody-Corbett and Gilbert, 1992b; Chauhan-Patel and Spruce, 1998; Spruce and Moody, 1992). A second current, I_{K1} (sometimes called I_A), activated with similar kinetics to I_K , but showed rapid inactivation (Ribera and Spitzer, 1990; Moody-Corbett and Gilbert, 1992a;

Chauhan-Patel and Spruce, 1998; Spruce and Moody, 1992). The inactivation kinetics of I_{K1} best fit by two time constants, about 4 ms and 70 ms (Moody-Corbett and Gilbert, 1992a). The third voltage gated K^+ current, I_o (Spruce and Moody, 1992) activated at potentials slightly more positive than I_K or I_{K1} , activated very rapidly with depolarization, and showed no inactivation. The density of this current was very low (1 pA/pF), and did not increase in density with time in culture as did I_K and I_{K1} (Spruce and Moody, 1992).

In addition to the voltage activated K^+ currents, other outward K^+ currents have been described in skeletal muscle. These are the Ca^{++} activated K^+ currents (Stanfield, 1983), which are regulated by both intracellular Ca^{++} concentration. Two types of Ca^{++} activated K^+ currents have been described. One class, the small conductance, voltage insensitive channel (SK_{Ca}), is sensitive to the bee venom apamin, but not TEA (Blatz and Magleby, 1986; Romey and Lazdunski, 1984). The other class, the large conductance, voltage sensitive channel (BK_{Ca}) is insensitive to apamin, but blocked by TEA and the scorpion venom charybdotoxin (Pallotta *et al.*, 1981; Wu and Haugland, 1985; Miller *et al.*, 1985). The voltage activated K^+ currents and Ca^{++} activated K^+ currents have been hypothesized to play distinct roles in muscle electrical activity: the role of the non-inactivating K^+ current is to shorten the duration of the action potential, and the role of the Ca^{++} activated current is to maintain an afterhyperpolarization and thus attenuate electrical activity and action potential

frequency during periods of metabolic stress when intracellular calcium is high (Fink *et al.*, 1983; Barrett *et al.*, 1981; Pallotta *et al.*, 1981).

Another class of K^+ currents which has been described in skeletal muscle is the ATP-sensitive K^+ current (I_{KATP} ; Allard and Lazdunski, 1992; Spruce *et al.*, 1987, 1989; Vivaudou *et al.*, 1991; Castle and Haylett, 1987). This current is voltage insensitive, however it is activated when the concentration of intracellular ATP is reduced (for review, see Standen, 1992). As such, I_{KATP} is normally only active under conditions of metabolic stress. I_{KATP} is proposed to protect the muscle fibre during anoxia or fatigue by lowering excitability and reducing Ca^{++} entry through voltage gated Ca^{++} channels (Castle and Haylett, 1987).

Thus it became obvious that the ionic currents expressed in muscle were more complex than the I_K and I_{Na} described by the Hodgkin and Huxley models. Clear roles have not been elucidated for the two kinetically distinct voltage gated K^+ currents observed in skeletal muscle. The major aim of my thesis research was to gain a greater understanding of the voltage activated K^+ in skeletal muscle. To this end, specific aims included determining if different K^+ currents had unique spatial distributions similar to Na^+ channels in skeletal muscle (described below) and isolation of cDNA sequences which may encode K^+ channels expressed in skeletal muscle. Detailed knowledge of the spatial distributions and molecular identities of the channels responsible for the current types in skeletal muscle would help us understand their physiological roles.

1.3.2 Spatial distribution of ionic currents in skeletal muscle

Adrian and colleagues (Adrian *et al.*, 1970a,b) were the first to suggest that the spatial localization of the inactivating voltage activated K^+ current, sustained current and inward rectifier channels could play an important role in determining the electrophysiological properties of skeletal muscle. They proposed that the non-inactivating voltage activated K^+ current was located in the surface membrane, while the other two were located in the T-tubular system. Thus, a seed of thought was planted: the electrophysiological characteristics of skeletal muscle (and indeed, other excitable cells) are not spatially uniform. Soon after the report by Adrian and colleagues, another group reported that action potentials initiated near the NMJ had a rise time about 20% faster than those initiated away from the NMJ (Nastuk and Alexander, 1973). They proposed that there were more Na^+ channels in the area of the endplate. The same phenomenon was observed by Thesleff *et al.*, (1974). These were important discoveries considering that they were inconsistent with the "fluid mosaic model" of proteins in biological membranes (Singer and Nicolson, 1972).

The presence of an uneven distribution of Na^+ channels in the membrane of skeletal muscle was confirmed electrophysiologically with a vibrating electrode technique (Betz *et al.*, 1984), and with a loose patch clamp technique (Almers *et al.*, 1983;1984; Beam *et al.*, 1985; Caldwell *et al.*, 1986; Roberts *et al.*, 1984; 1986;1987) as described below. The early loose patch clamp studies

revealed that Na⁺ channels were not evenly distributed in the muscle membrane: they showed up to ten-fold differences in lateral distributions, and thousand-fold differences in density near to and distant from the NMJ.

The clustering of Na⁺ channels at the NMJ was also observed with fluorescently labeled scorpion toxin (Angelides, 1986) and immunocytochemistry (Haimovich *et al.*, 1987). In mature mammalian skeletal muscle where the post synaptic membrane shows deep folds, immunoelectron microscopy (Flucher and Daniels, 1989) and use of radiolabeled scorpion toxin (Boudier *et al.*, 1992) have revealed a high density of Na⁺ channels in the troughs of the post-synaptic folds.

The clustering of Na⁺ channels at the NMJ is important to ensure that a synaptic event can trigger enough current to initiate an action potential in the muscle cell thus leading to activation of the contractile machinery of the cell (Caldwell, 2000).

The loose patch clamp method was a particularly useful method because it allowed a direct measurement of Na⁺ currents at various positions along the muscle cell. The technique is exactly as the name implies: patch clamp recording (Hamill *et al.*, 1981) with an electrode that forms a physically and electrically loose seal, not a tight one (reviewed in Roberts and Almers, 1992). Since the electrodes do not form adhesive seals with the cell membrane, many recordings can be performed on the same cell without causing any damage. The loose patch clamp has been used to estimate the perisynaptic density of

Na⁺ channels in skeletal muscle at about 2000/ μm^2 , and the extrajunctional density at about 100/ μm^2 (Caldwell *et al.*, 1986; Milton *et al.*, 1992).

A report using the loose patch clamp (Almers *et al.*, 1983) stated that there was no correlation between Na⁺ current and K⁺ current on muscle cells, and another report stated that there was no correlation between K⁺ currents and distance from the NMJ (Roberts, 1987). Since then, little attention has been paid to examining the spatial distribution of K⁺ channels in muscle. However, for a number of technical reasons it is likely that these early studies would not have observed any correlation between K⁺ channel and Na⁺ channel distributions if it did exist. While the loose patch clamp is an ideal tool to examine Na⁺ currents, it is a poor one to use to examine K⁺ currents. When a patch of membrane is depolarized to very positive potentials the leakage of current under the rim of the loose patch clamp pipette becomes significant. This causes an uncontrolled potential that can activate Na⁺ channels under the pipette rim and adjacent membrane and make analysis of slower currents such as the voltage activated K⁺ current difficult (Almers *et al.*, 1983; Walter Stuhmer, personal communication). This problem may be exacerbated by the fact that the muscle preparations were not thoroughly cleaned of their thick basal laminae. Moreover, these early experiments were primarily concerned with Na⁺ current, and all the voltage steps were extremely short. K⁺ current was measured after four ms of depolarization, and this time frame was probably not adequate to fully

activate all K^+ channels and allow proper examination of kinetics of the K^+ currents (Adrian *et al.*, 1970). These experiments offered little insight into the spatial distribution of K^+ channels in skeletal muscle, or how membrane repolarization kinetics in areas of high excitability might differ from kinetics in areas of lower excitability.

There is however some evidence to suggest that K^+ channels may indeed show a specific distribution in skeletal muscle membrane. One loose patch clamp study (Almers *et al.*, 1984) showed a mild correlation ($r = 0.7$) between K^+ and Na^+ currents in human skeletal intercostal muscle. Furthermore, a report by Roberts *et al.*, (1986) and Weiss *et al.*, (1986) used a photobleaching technique to monitor Na^+ and K^+ channel mobility, and they reported that K^+ and Na^+ channels have very low mobility in the skeletal muscle sarcolemma. Taken together, these two studies provide support for the notion that K^+ channels are anchored in the membrane with some specialized distribution that plays a role in determining the electrophysiological characteristics in muscle.

Other synapses have recently been shown to have post synaptic clusters of K^+ channels. For example Alonso and Widmer (1997) showed by immunoelectron microscopy, that Kv4.2, which is known to encode an inactivating K^+ current, is clustered beneath sites of synaptic contacts in rat supraoptic neurons. Yazulla and Studholme, (1999) also showed by immunoelectron microscopy that Kv4.2 channel and the AMPA-glutamate receptor (GluR4) are

co-localized on the dendrites of OFF-bipolar cells innervating photoreceptor cells in goldfish retina. Other studies using the combined techniques of *in situ* hybridisation and immunocytochemistry have provided indirect evidence of specific, subcellular localization of voltage gated K^+ channels. For example, Sheng *et al.*, (1992) report targeting of Kv4.2 to somatodendritic compartment of hippocampal cells and cerebellar granule cells. Similarly, Wang *et al.*, (1994) suggest that Kv1.1 and Kv1.2 may be present in discrete and multiple subcellular locations including proximal dendrites in mouse central neurons. Other recent studies have shown that voltage gated K^+ channels are also clustered in the postsynaptic muscle membrane of the larval *Drosophila* NMJ. Four types of K^+ currents were found in this muscle, including a non-inactivating voltage activated K^+ current, two different Ca^{++} dependent K^+ currents, and I_A , (Salkoff and Wyman, 1983; Broadie and Bate, 1993b). Tejedor *et al.*, (1997) have found that the *Shaker* gene product, mediating the I_A , is localized to the post synaptic membrane beneath Type 1 synaptic boutons.

Given that the accumulation of AChRs and Na^+ channels serves to ensure that a synaptic event is translated to an action potential in the muscle membrane, it is logical to hypothesize that there would also be an accumulation of K^+ channels near the NMJ to ensure a rapid repolarization of the muscle membrane after a depolarization. Without a strong repolarizing K^+ current in the area of the endplate the Na^+ channels may be slow to make the transition from

the inactivated state to the closed state after opening. Indeed, a suggested role of the postsynaptic clustering of I_A in *Drosophila* muscle is to control muscle activity in response to synaptic activity (Tejedor *et al.*, 1997). Such regulation may be required for normal synaptic development (Budnik *et al.*, 1990; Broadie and Bate, 1993a; Jarecki and Keshishian, 1995). Most of the studies above which describe a postsynaptic localization of K^+ channels used immunocytochemical techniques. Though highly informative, these studies do not directly study the function of such localization. In contrast, the *Xenopus* NMJ in culture offers a chance examine function of postsynaptic ion channel distribution. Based on this, and the studies describing postsynaptic localization of K^+ channels, I thought that the spatial distribution of K^+ channels in vertebrate skeletal muscle should be re-examined (Chapter 2). Furthermore, the molecular diversity of the K^+ channels in other systems together with the known K^+ currents in skeletal muscle suggested that multiple channels underlie the expression of K^+ current in skeletal muscle. Understanding the roles of these specific currents in skeletal muscle required identifying the channels which were expressed (Chapter 3).

1.4 *Xenopus* as a model system

Embryonic myotomal muscle from the African Clawed Frog, *Xenopus*

laevis, provided a useful model for studying the distribution of K^+ channels relative to sites of synaptic contact. *Xenopus* is one of the most commonly used models for studying development of electrical excitability and it had some clear general advantages over other models, such as mouse, rat, or chick. It is easy and inexpensive to produce large numbers of identically aged *Xenopus* embryos at one time. The development of *Xenopus* is well characterized in the normal tables of Nieuwkoop and Faber (1967), and occurs totally outside of the body. The developmental importance of numerous proteins has been experimentally tested by injection of cRNAs (Jones *et al.*, 1995, Jones and Ribera, 1994) or antisense RNA (Vincent *et al.*, 2000) into freshly fertilized embryos. Embryos can be easily subjected to experimental manipulation at various stages of development from fertilization through neurulation and innervation of muscle by motor neurons.

There are also some specific factors which make *Xenopus* an attractive system to use in studying ion channel distributions in nerve contacted skeletal muscle. Firstly, culturing muscle and spinal cord neurons from *Xenopus* embryos is straightforward and well established in the literature (Anderson *et al.*, 1977; Anderson and Cohen, 1977; Cohen, 1980). The muscle and nerve could each be isolated free of contamination from each other and other cell types such as fibroblasts, so antimitotic agents were not required. Furthermore, the cells are fairly small, and myocytes grow at low densities, so a large number could be

plated on one culture dish without contacting each other (Anderson *et al.*, 1977).

Secondly, in culture, many of the myotomal cells contacted by spinal cord neurites become functionally innervated in less than a day (Anderson *et al.*, 1977; Anderson *et al.*, 1979; Peng *et al.*, 1979). The muscle cells show aggregations of AChRs along pathways of nerve contact by one day in culture (Anderson and Cohen, 1977; Anderson *et al.*, 1977), and these sites form within hours (Cohen *et al.*, 1979). Almost all muscle cells with such aggregations of AChRs showed synaptic activity (Anderson *et al.*, 1979). In one day old cultures specializations such as presynaptic clusters of vesicles, basal lamina like material in the cleft, and electron dense post-synaptic sarcolemma with underlying filamentous projections (Weldon and Cohen, 1979), and accumulations of acetylcholinesterase (Moody-Corbett and Cohen, 1982; Moody-Corbett *et al.*, 1982) and also identifiable. This development closely parallels the development of the NMJ of myotomal muscle *in vivo* (Chow and Cohen, 1983; Kullberg *et al.*, 1977; Nieuwkoop and Faber, 1967; Cohen, 1980). Moreover, the development of post-synaptic specializations on the muscle cells in culture was easily traced in living cultures by examining the binding of tetramethylrhodamine-labeled α -bungarotoxin to aggregations of AChRs (Anderson and Cohen, 1974).

A third factor making *Xenopus* nerve-muscle co-cultures attractive for the study of postsynaptic localization of ion channels is that the ionic currents in

Xenopus muscle cells in culture have been well described, so a detailed preliminary analysis to identify the currents present was not necessary. The muscle cells are known to express only a few ionic currents. In addition to the three K^+ currents I_K , I_{K1} and I_o , the muscle cells express: a single TTX sensitive voltage gated Na^+ current, or possibly two TTX sensitive currents with very similar single channel conductances and activation properties (DeCino and Kidokoro, 1985); two voltage gated Ca^{2+} currents, one having slow activation kinetics and sensitivity to dihydropyridines, and the other having rapid activation/inactivation kinetics and insensitivity to dihydropyridines (Moody-Corbett *et al.*, 1989; Moody-Corbett and Virgo, 1991) and two inward rectifier currents, each having different single channel properties (Moody-Corbett and Gilbert, 1991; Hancock *et al.*, 1996). *Xenopus* muscle cells in culture do not express a resting Cl^- conductance (Spruce and Moody, 1992), which would have made analysis of K^+ currents difficult. *Xenopus* muscle cells in culture do express an ATP-sensitive channel, but this is only detectable under non-physiological conditions (Honore and Lazdunski, 1995).

Fourth, a number of K^+ channel cDNA clones have been isolated from *Xenopus*. Ribera's and Spitzer's groups have identified a number of Kv channels which are expressed during neuronal development. They have cloned cDNAs encoding the α subunits Kv1.1, Kv1.2, Kv 2.1 Kv2.2 Kv3.1 (Ribera, 1990; Ribera and Nguyen, 1993; Burger and Ribera, 1996; Gurantz *et al.*, 2000),

Kv4.3 (Lautermilch, N.J. and Spitzer, N.C., unpublished, accession # U89265), and two β subunits Kv β 2, and Kv β 4 (Lazaroff *et al.*, 1999). Their studies have indicated that all of these were expressed in neurons, and Kv2.2 and Kv β 2 were expressed in muscle (Lazaroff *et al.*, 1999; Burger and Ribera, 1996).

Finally, the *Xenopus* nerve-muscle co-culture is an ideal (and appropriate) model system to use in studying the relationships between voltage gated K^+ and Na^+ channels because there is much evidence that expression of K^+ and Na^+ channels are co-regulated and are closely linked to the presence of spinal cord neurons. DeCino and Kidokoro, (1985) reported that culturing muscle cell with neural tube did not change the activation or inactivation kinetics of Na^+ currents, but did induce the expression of a second class of Na^+ channel with a slightly lower single channel conductance, and caused an increase in the overall Na^+ channel density in the cell membrane. This effect may have been independent of neurite contact on the muscle cell. Prabhakar *et al.*, (1996) reported that co-culture of myocytes with spinal cord neurons or culture in nerve-conditioned media increased the proportion of muscle cells that expressed Na^+ current after one day in culture, and increased the Na^+ current density of those cells that did express Na^+ current. Development of K^+ currents in muscle cells in culture also appeared to be influenced by the presence or absence of neurons. Ribera and Spitzer (1991) reported that myocytes that were grown in muscle enriched (essentially aneural) cultures showed K^+ currents with slower activation

kinetics, and lower K^+ current densities up till about 48 h in culture. Although in this study Ribera and Spitzer recognized the presence of I_{K1} in muscle cells (they term the I_{K1} the “A current”), they based all K^+ current density measurements on the I_K component and did not investigate the effect of co-culture with neurons on I_{K1} . Using single channel patch clamp analysis, Ernsberger and Spitzer, (1995) examined the effect of the presence of spinal cord neurons on the development of inactivating and non-inactivating K^+ current in *Xenopus* skeletal muscle. They found that channels mediating an inactivating K^+ current undergo a six-fold increase in density when neurons were co-cultured with the muscle, whereas they observed a decrease in density if neurons were omitted from the culture. Conversely, channels mediating the slowly inactivating current (they are essentially non-inactivating, having time constants up to 2000 ms) underwent a two-fold decrease in density during the same period in the presence or absence of neurons.

Na^+ and K^+ channel expression are related in embryonic *Xenopus* myocytes. Linsdell and Moody, (1994) cultured muscle from embryos which had been injected with rat brain Na^+ channel RNA at stage 1. The presence of exogenous Na^+ channels allowed muscle cells from injected embryos to potentially undergo action potentials much sooner than myocytes from uninjected control embryos. Linsdell and Moody observed an increase in expression of both the outward K^+ current, and inward rectifier current in

myocytes from injected embryos. They also showed that culturing myocytes in TTX inhibited the development of the outward K^+ current. However they did not separately analyze the effect of the exogenous Na^+ current on I_{IK} or I_K , nor did they analyze the single channel properties to determine whether the population of K^+ channels had changed, or if the density of the existing types of K^+ channels had simply been increased. However the results of this study suggested that expression of K^+ channels in developing *Xenopus* myocytes is also regulated by electrical activity. Spruce and Moody, (1995) also reported that overexpression of a mouse brain K^+ channel in muscle appeared to accelerate the developmental time of appearance of the endogenous voltage gated K^+ currents as well as increase the density of the voltage gated K^+ currents and inward rectifier currents in *Xenopus* myocytes.

The spatial distribution of K^+ and Na^+ channels in the membrane of *Xenopus* myocytes in culture was examined in order to determine if spatial expression of Na^+ and K^+ currents were related (Chapter 2). In order to more closely examine the spatial distribution of K^+ channels in these muscle cells, and to determine the importance of specific K^+ currents to the physiological properties of the postsynaptic apparatus, the molecular identities of the K^+ channels which are expressed must be known. Towards this, the molecular identities of three channels which were expressed in *Xenopus* muscle was determined using molecular biological techniques (Chapter 3).

1.5 Specific Aims

1.5.1 Aim of Chapter 2

Several factors led to the hypothesis that K^+ channels may be localized near the synapse in postsynaptic muscle membrane: (1) loose patch clamp studies stating that there is no specialized spatial distribution of K^+ channels in muscle membrane may not have been able to effectively measure K^+ currents (Almers *et al.*, 1983; Roberts, 1987), (2) postsynaptic localization of K^+ channels has been reported for a number of systems including central synapses (Alonso and Widmer, 1997) and *Drosophila* NMJ (Tejedor *et al.*, 1997), (3) Na^+ channels are clustered at high density near the NMJ, and a complimentary distribution of K^+ channels could enable more rapid recovery of Na^+ channels from inactivation, thus increasing the possible rate of firing of action potentials.

The aim of the experiments described in Chapter 2 was to examine the spatial distribution of K^+ channels in the sarcolemma of embryonic *Xenopus* muscle cells in culture with respect to synaptic sites. This study used a variation of the tight seal cell attached patch clamp method (Hamill *et al.*, 1981) called the macropatch technique to map the K^+ channel density on muscle cells at various distances away from nerve contact. The method is identical to that used for single channel recording, except the patch electrodes are larger. A typical

electrode for single channel recording has a tip diameter 1 μm or less, whereas the tip diameter of a macropatch electrode may be $> 2\mu\text{m}$. Patch electrodes with larger tips allow recording from larger areas of muscle, and thus more accurate density estimates. By virtue of the gigohm seals established between electrode and cell membrane, the technique does not suffer from the problem of current leaking under the rim of the electrode as does the loose patch clamp.

1.5.2 Aim of Chapter 3

Numerous studies have shown that there are at least two components of the voltage gated K^+ currents in embryonic *Xenopus* myocytes in culture, and thus there are probably at least two distinct subtypes of K^+ channels, however the molecular identities of these channels are presently unknown. Indeed, there is no data correlating the electrophysiological properties of skeletal muscle with the spectrum of K^+ channels expressed in skeletal muscle. An interesting study from Lesage *et al.*, (1992) showed that the mRNA for many Kv1 subtypes is expressed in developing rat skeletal muscle, although the contribution of these channels to the electrophysiological properties of skeletal muscle was not assessed. Furthermore, of all the Kv α channels isolated by Ribera's group and Spitzer's group from *Xenopus* neurons, only one, XKv2.2 has been shown to be expressed in embryonic muscle. Therefore, although there is substantial information about the K^+ currents in *Xenopus* muscle during development, there is little information about the channels that are responsible for I_{K} and I_{K} , or how

they are regulated. The aim of Chapter 3 was to (1) isolate cDNAs encoding voltage gated K^+ channel proteins from *Xenopus* muscle, (2) express the K^+ channel cDNAs in a heterologous expression system to examine the K^+ currents produced by the channels and (3) examine the expression of these K^+ channel genes during early embryonic development and during differentiation of skeletal muscle.

The isolation of cDNAs encoding K^+ channels from *Xenopus* muscle, and the characterization of the currents produced by these channels will provide tools for addressing questions about the functional significance of different types of K^+ currents in skeletal muscle, and the roles they may play in the development of the neuromuscular synapse. Understanding the roles of K^+ channels during the development of the NMJ may lead to a greater understanding of the physiology of central synapses.

Co-authorship statement

Chapter 2 is a manuscript which has been published in the journal *Pflügers Archiv*, 437: 895-902. Dr Moody-Corbett designed the initial research proposal, while the author of this thesis, William Mark Fry, carried out all practical aspects of the research and the data analysis. The manuscript was prepared as a combined effort from Dr Moody-Corbett and the author of this thesis.

Chapter 3 is a modification of a manuscript which has been accepted for publication in the journal *Molecular Brain Research*. The key difference between the accepted manuscript and Chapter 3 is the addition to Chapter 3 of preliminary data analyzing the electrophysiological properties of the channels encoded by the XKv1.2', XKv1.4 and XKv1.10 channels expressed in HEK 293 cells. The author of this thesis designed the research proposal, and the author of this thesis carried out most practical aspects of the research and data analysis. Dr Frances Moody-Corbett carried out some of the HEK 293 cell transfections and patch clamp recordings, and Dr Karen Mearow carried out the rest of the HEK cell transfections. The molecular biological experiments were carried out in the lab of Dr Gary Paterno. The manuscript submitted to the journal *Molecular Brain Research* was prepared as a combined effort from Dr Moody-Corbett and the author of this thesis, with critical comments from Dr Gary Paterno.

Chapter 2

Localization of Sodium and Potassium Channels at Sites of Nerve-Muscle Contact in Embryonic *Xenopus* Muscle Cells in Culture

2.1 Introduction

Electrical excitability is determined by both the complement of ion channels and the distribution of these ion channels in the membrane. At the vertebrate adult neuromuscular junction (NMJ) the receptors to the neurotransmitter acetylcholine (ACh) are localized at the crests of the post-synaptic folds and in the troughs there is a localization of the tetrodotoxin (TTX)-sensitive sodium channels (Catterall, 1992; Grinnell, 1995). The presence of these two classes of channels assures that the synaptic event will trigger a membrane action potential. In contrast, very little information is available on the spatial distribution of the potassium channels which are involved in the repolarization of the membrane following an excitatory event.

Regulation of membrane repolarization in skeletal muscle involves two types of outward potassium currents (Duval and Leoty, 1980a): current which inactivates during maintained depolarizations (I_{K1}) and current which shows very little inactivation (I_K). The spatial distribution of the channels which mediate these potassium currents has not been examined in skeletal muscle. Using the loose patch clamp technique, and brief voltage steps, 'hotspots' of potassium current density have been identified in a variety of adult muscle membrane preparations (Almers *et al.*, 1984; Almers *et al.*, 1983; Roberts, 1987). The potassium currents tended to be small compared with the sodium currents under

study and only in human muscle was a weak relationship between I_{Na} and potassium current reported (Almers *et al.*, 1984). In addition, because of the brief duration of the voltage steps the different classes of potassium currents, based on inactivation properties, were not distinguished. More recently, using immunohistochemical techniques, it has been found that potassium channels form clusters associated with the post-synaptic specializations at certain excitatory central nervous system synapses (Alonso and Widmer, 1997) and at the NMJ of *Drosophila* larval muscle (Tejedor *et al.*, 1997). Given these findings we were interested in re-examining the relationship between sites of nerve-contact and I_{Na} and potassium current distribution in vertebrate skeletal muscle. We have used tight-seal macro-patch recordings on embryonic *Xenopus* muscle cells which were contacted by spinal cord neurons in culture. This preparation offers the advantage of forming synaptic connections which have localizations of post-synaptic specializations (Cohen, 1980). In addition, the sites of nerve-contact are easily accessible for electrophysiological study using a phase contrast inverted microscope. Further, both I_K and I_{K1} (Ernsberger and Spitzer, 1995; Moody-Corbett and Gilbert, 1992a,b; Spruce and Moody, 1992) and TTX-sensitive I_{Na} (DeCino and Kidokoro, 1985) are present in these muscle cells in culture.

The results indicate that I_{Na} and I_{K1} are co-localized near sites of nerve-muscle contact. The potassium current densities, when associated with I_{Na} , are

significantly higher than elsewhere in the membrane and suggest that the channels, which mediate this current, are clustered with sodium channels in the post-synaptic region.

2.2 Methods

Nerve-myocyte co-cultures were prepared from stage 19 - 22 *Xenopus* embryos (Nieuwkoop and Faber, 1967). Back portions were dissected away from the rest of the embryos using stainless steel insect pins in dissecting medium containing 2/3 Leibovitz L15 medium, 0.5% dialyzed horse serum, 0.01 mg/ml gentamycin, 5 units/ml nystatin. The back portions were placed in dissecting medium containing 0.5 - 1 mg/ml collagenase for 10 - 20 m and the myotomal muscle layer dissected away from the spinal cord, notochord, and skin. Myotomes and spinal cords were retained and separately placed in Ca^{++} -/Mg $^{++}$ free Ringer solution for 30 m and allowed to further dissociate. Both muscle and nerve cells were then briefly placed in plating medium (2/3 Leibovitz L15 medium, 5% dialyzed horse serum) and the cells were co-cultured on collagen (Sigma Chemical Co., Scarborough ON) and ECL (Upstate Biotechnology, Lake Placid, NY) treated coverslips in culture medium (2/3 Leibovitz L15 medium, 0.5% dialyzed horse serum). The cultures were stored at room temperature in the dark until use (1-3 days). Leibovitz L15 nutrient

medium, antibiotics, and collagenase were all purchased from GIBCO-BRL (Burlington, ON).

Macroscopic currents were recorded from muscle cells which were contacted by spinal cord neurons (one current recording per cell). The cells were visualized at 400X magnification under phase contrast optics (Zeiss IM-35). In order to confirm the presence of synaptic contacts on living *Xenopus* muscle cells in culture three cultures were stained tetramethylrhodamine-labeled α -bungarotoxin (Molecular Probes Inc., Eugene, OR) and examined, using fluorescence optics, for the localization of ACh receptors at sites of nerve-contact.

Currents were obtained from tight seal ($> 1 \text{ G}\Omega$), cell-attached macro-patches using an EPC7 List patch clamp amplifier (Medical Systems Corp, Greenvale NY). The macro-patch electrodes were constructed from Drummond 100 borosilicate glass (Fischer Scientific) or TW150-F thin wall borosilicate glass (World Precision Instruments Inc, Sarasota FL) and filled with extracellular recording solution (described below). The electrodes were pulled on a PP-83 two stage vertical puller (Narashige, Japan), coated with Sylgard (Dow Corning, Midland MI), and firepolished. The diameters of the electrode tips, measured by visualization at 630X magnification, ranged from 2 to 6 μm , with resistances between 3.3 and 1 M. The electrodes were bent near the tip to allow a vertical approach to the muscle cells and were placed on the muscle membrane using a

motorized micromanipulator attached to the microscope. A graticule in the eyepiece allowed measurement of distances between the electrode and nerve contact on muscle membrane to within 2.5 μm .

The extracellular recording solution in the bath contained (in mM): 143 NaCl, 2 KCl, 1.2 CaCl_2 , 1 MgCl_2 , 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.4). In experiments determining potassium reversal potentials, the potassium concentration was increased, and the sodium concentration was reduced by an equimolar amount. In experiments determining sodium reversal potential, TRIS chloride (TRIS-Cl) replaced sodium chloride when the sodium concentration was reduced. In order to eliminate the contribution of either chloride or calcium ions to the recorded currents an extracellular recording solution of the following composition was used (in mM): 143 mM Na^+ acetate, 2 mM K^+ acetate, 1 MgSO_4 , 10 HEPES (pH 7.4).

In a single experiment recordings were obtained in the presence or absence of the sodium channel blocker TTX. Thirteen macro-patches were examined with 5 μM TTX added to the recording electrode and the results compared with recordings from 12 macro-patches obtained in the absence of TTX. To increase the probability of recording from a membrane patch which had I_{Na} , recordings were made within 12.5 μm of nerve-muscle contact (see results). The recording electrodes were alternated such that a recording was made with TTX present and the next without TTX present.

Pclamp software (version 5.01 and 6.1, Axon Instruments, Inc., Foster City, CA) was used for voltage control, data acquisition and data analysis. Macro-patches were held at rest (*i.e.*, no applied potential) and subjected to a series of 100 ms depolarizing pulses to record slow currents, and 20 ms pulses to record fast currents. The currents obtained during the 100 ms pulses were leak subtracted off-line, and the currents obtained during the 20 ms pulses were leak subtracted with a P/4 routine online. The data were digitized at 10 or 100 kHz, respectively, and filtered at the patch-clamp amplifier at 3 kHz.

Tight seal macro-patches generally formed in less than a minute after slight suction was applied to the pipette upon contact with a muscle cell. The surface area of the membrane in the macro-patch was assumed to be a hemisphere (Matsuda and Stanfield, 1989) with a diameter equal to the diameter of the pipette tip. In only a few cases did the macro-patch appear to not be a hemisphere, and these were not included. This observation is in agreement with Milton and Caldwell (1990) who rarely observed blebs in non-enzymatically treated muscle. Current densities were calculated by dividing the observed current by the calculated area of the macro-patch and are expressed as $\text{pA}/\mu\text{m}^2$.

Resting membrane potentials (RMPs) were determined in 18 muscle cells by recording RMP in current clamp mode of the patch clamp. The RMP was recorded immediately after gaining access to the cell interior in muscle cells bathed in standard external recording solution. In this case the patch electrodes

were filled with (in mM): 90 KCl, 5 MgCl₂, 1 1,2-bis(2-aminophenoxy)ethanesulfonic acid (BAPTA), 10 HEPES (pH 7.4) and had resistances of 8 - 10 M Ω . In 7 cells RMP was determined using a DUO-773 microelectrode amplifier (World Precision Instruments Inc., Sarasota, FL) and intracellular microelectrodes, filled with 3 M KCl and having resistances of 50 M Ω .

2.3 Results

In culture *Xenopus* spinal cord neurites contact muscle cells on both the top and bottom surfaces (*i.e.*, away from and touching the coverslip, respectively) and may contact more than one muscle cell (Cohen *et al.*, 1987). Furthermore, one muscle cell may have numerous nerve contacts. In this study only those muscle cells with a single nerve contact on a top surface were used in the macro-patch experiments. Current recordings were obtained at various distances from these sites of nerve contact on muscle cells grown for 1 to 3 days in culture.

The average RMP of nerve-contacted muscle cells measured using whole cell recordings and the patch clamp amplifier in current clamp mode was -81 ± 2.9 mV ($n = 18$). This was similar to the average RMP measured using a conventional microelectrode amplifier (-77 ± 5 mV, $n = 7$). Based on these

measurements the membrane potential at zero applied potential during the macroscopic current recording experiments, in standard solutions, was estimated to be -80 mV and the membrane potentials reported below refer to the final membrane potentials calculated from this RMP.

Membrane depolarization is known to activate five different macroscopic currents in *Xenopus* muscle cells in culture. Two of these currents are mediated by potassium ions and differ in the rate of inactivation. They have been designated I_K , for the non-inactivating outward current, and I_{IK} , for the inactivating outward current (Ernsberger and Spitzer, 1995; Moody-Corbett and Gilbert, 1992a,b). Two of the inward currents are mediated by calcium ions and show properties similar to the L-type and T-type calcium currents described in other muscle preparations (Moody-Corbett *et al.*, 1989). Finally, a TTX-sensitive inward sodium current has been identified in these muscle cells in culture (DeCino and Kidokoro, 1985). In the present study membrane depolarization resulted in the appearance of three types of current: one inward current, identified as I_{Na} and two outward currents, identified as I_K and I_{IK} .

Ionic currents were recorded from 133 macro-patches at varying distances from the sites of nerve contact. In 51 of the patches there were no detectable currents upon membrane depolarization, while in 82 macro-patches inward and/or outward currents were apparent. There was a higher probability of finding currents in membrane macro-patches localized near the site of nerve

contact, although macro-patches which did not have a detectable current and macro-patches with one or more type of current were present in all areas of the membrane (Figure 2.1).

2.3.1 Identity of the Ionic Currents

In 35 of the macro-patches rapidly activating and inactivating inward currents were apparent (Figure 2.2A). These currents activated near -40 mV and the peak inward current was recorded near 0 mV (Figure 2.2B). In standard external recording solution (sodium concentration 143 mM) I_{Na} reversed at $+77 \pm 3.6$ mV ($n = 7$), positive to the calculated reversal potential ($E_{Na} = +67$ mV) assuming an intracellular sodium concentration of 10 mM (Spitzer, 1976). In 70 mM external sodium I_{Na} reversed at $+53 \pm 6.3$ mV ($n = 5$), not significantly different from the calculated reversal potential ($E_{Na} = +49$ mV). These results suggested that the inward currents were mediated by sodium ions. Two additional experiments were conducted to clarify that the currents were I_{Na} . (1) The inward currents were absent when TTX was included in the recording pipette ($n = 13$ macro-patches) whereas inward currents were seen in five of 12 macro-patches recorded in the absence of TTX. These recordings were obtained near sites of nerve contact, which was found to be the preferential location of macro-patches with I_{Na} (see below). The presence of TTX in the electrode did not alter the appearance of the outward currents. (2) The rapidly activating and inactivating inward currents were similar in the presence or

Figure 2.1 Percentage of macro-patches at each distance from the site of nerve contact which had inward and/or outward current. The number of patches examined at each distance is indicated above each point.

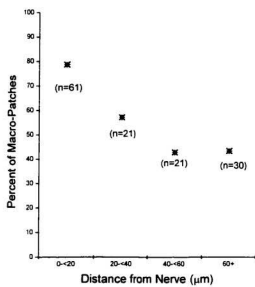
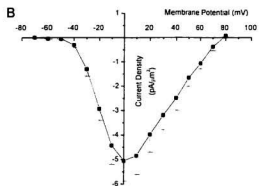
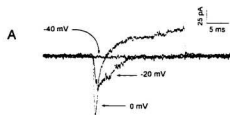


Figure 2.2 Voltage gated Na^+ current. A. Superimposed current traces showing inward I_{Na} recorded from a macro-patch using the P/4 leak subtraction protocol. The macro-patch was $10 \mu\text{m}^2$ in area and was $5 \mu\text{m}$ from the nerve-contact. The potential to which the macro-patch membrane was stepped is indicated for each trace. B. Plot of the mean (and standard errors) I_{Na} density versus membrane potential from 13 macro-patches.

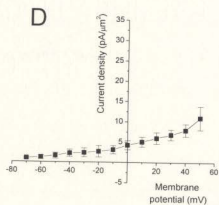
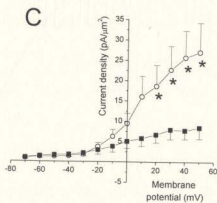
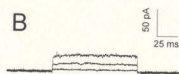
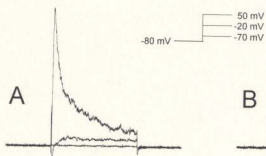


absence of calcium ions in the extracellular recording solution ($n = 9$ macro-patches). Taken together, therefore, these results indicate that the inward currents seen during membrane depolarization in these macro-patches were carried by sodium ions.

Outward currents were found in 81 macro-patches. Based on the following results, and the similarity of these macroscopic currents to potassium currents recorded from whole *Xenopus* muscle cells in culture, the currents were assumed to be carried by potassium ions. The currents were classified as inactivating (I_{ik}) if they decreased more than 20% during the 100 ms voltage step and non-inactivating (I_k) if they changed less than 20% during the voltage step (Figure 2.3A and B). There was a higher proportion of macro-patches with I_{ik} (72%) than I_k (28%), but no identifiable differences between cells expressing each current type. The inactivation of I_{ik} at 50 mV was fit with a single exponential function giving an average $\tau = 14.6 \pm 9$ ms ($n = 47$), similar to that reported by Ernsberger and Spitzer (Ernsberger and Spitzer, 1995) based on single channel recordings. Both I_{ik} and I_k activated near -40 mV, increasing in amplitude with increased membrane potential (Figure 2.3C and D). Because of the low frequency of occurrence of I_k , only reversal potentials for I_{ik} were measured. In 2 mM extracellular potassium the average reversal potential of I_{ik} was slightly positive to resting potential (-68 ± 1.6 mV, $n = 6$), similar to that reported previously from whole cell current recordings (Moody-Corbett and

Figure 2.3. Voltage gated K⁺ currents. A and B. Superimposed current traces from two macro-patches, one which contained I_{IK} (A) and the other I_K (B), which were depolarized from RMP to -70, -20 and +50 mV (voltage step protocol shown in the upper center). The macro-patch containing I_{IK} was 90 μm^2 in area and 3 μm from the nerve-contact. The macro-patch containing I_K was 67 μm^2 in area and located directly under the nerve-contact. C. Plot of the mean (and standard errors) potassium current density versus membrane potential for macro-patches which had I_{IK} and also I_{Na} (circles, n = 30) and I_{IK} without I_{Na} (squares, n = 24). D. Plot of the mean (and standard errors) potassium current density versus membrane potential for macro-patches which had I_K (squares, n = 17).

* The mean densities of I_{IK} , in patches containing I_{Na} , were significantly different than the mean densities of I_{IK} or I_K , in patches without I_{Na} , at the voltages indicated ($p < .05$, ANOVA, followed by Fisher's post-hoc test for multiple comparisons).



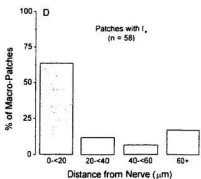
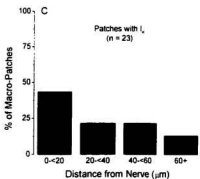
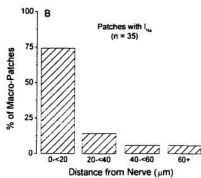
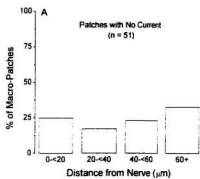
Gilbert, 1992a). Increasing extracellular potassium to 10 mM, the average reversal potential for I_{K1} shifted close to the potential predicted from the Nernst equation (-49.6 ± 2 mV, $n = 5$). Both I_{K1} and I_{K2} were present in chloride-free extracellular recording solution. Seventeen macro-patches were examined in chloride-free solution and 12 of these contained outward currents. I_{K1} was present in 10 macro-patches and two of the macro-patches contained I_{K2} . These results are similar to those obtained in chloride-containing extracellular recording solution and suggest that the outward currents recorded in the present study were mediated by potassium ions.

2.3.2 Spatial Distribution

The spatial distribution of macro-patches which contained no current, I_{Na} , I_{K1} and I_{K2} is shown in Figure 2.4. Macro-patches which showed no detectable current were found in all areas of the membrane, without a preferential distribution. However, macro-patches with one or more of the ionic currents tended to occur near the site of nerve contact (Figure 2.2 and 2.4).

Almost all of the macro-patches with I_{Na} occurred near sites of nerve-muscle contact (Figure 2.4B). Of the 35 macro-patches which contained I_{Na} , 26 macro-patches (74.3%) were located within 20 μ m of nerve contact and 31 macro-patches (88.6%) were within 40 μ m of the nerve contact (Figure 2.4B). In 32 (91.4%) of the macro-patches with I_{Na} , the inactivating potassium current, I_{K1} ,

Figure 2.4. Spatial distribution for macro-patches which contained (A) no current (white bars), (B) I_{Na} (cross-hatched bars), (C) I_K (black bars), and (D) I_{K1} (gray bars). The number of patches examined with no current or one of the three current classes is shown for each histogram and the percentage of macro-patches, in each category, is indicated relative to the distance from the nerve-contact.



was also present. In only one macro-patch was I_{Na} seen without an outward potassium current and in two macro-patches I_K was present along with I_{Na} .

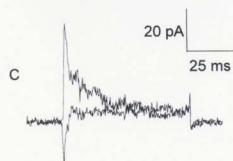
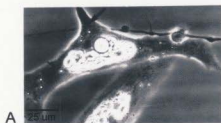
Macro-patches with potassium current were seen in all areas of the cell (Figure 2.4C and D), however a population of the macro-patches which contained I_{IK} had a distribution similar to I_{Na} . Of the 58 patches with I_{IK} 63.8% were found within 20 μm of the nerve contact and 76% within 40 μm of the nerve-contact (Figure 2.4D). The spatial distribution of I_{IK} was similar to that of I_{Na} (Figure 2.4B). Of the 23 macro-patches with I_K only 43.5% occurred within 20 μm of the nerve-contact and 65.2% within 40 μm of the nerve contact (Figure 2.4C).

The average density of the peak I_{IK} in the macro-patches which also contained I_{Na} was significantly higher than the average density of I_{IK} in macro-patches which did not contain I_{Na} (Figure 2.3C and D). At the most depolarized membrane potential tested (+50 mV) I_{IK} in membrane patches with I_{Na} was 27.1 ± 7.1 pA/ μm^2 ($n = 32$), compared with 8.7 ± 2.6 pA/ μm^2 ($n = 26$) in macro-patches which only contained I_{IK} . By comparison, the average density of I_{IK} in membrane patches without I_{Na} was very similar to the average density of I_K (9.2 ± 11.3 pA/ μm^2 , $n = 21$, at +50 mV). Since most of the membrane patches with I_{Na} and I_{IK} were found near the site of nerve-contact, it was possible that the increased density of I_{IK} was associated with the proximity to the nerve rather than association with I_{Na} . However, we found that the average current density of I_{IK} in

patches which did not contain I_{Na} , but were near the nerve contact ($<20\ \mu\text{m}$), was $6.9 \pm 2.7\ \text{pA}$ ($n = 12$), also significantly less than in patches containing I_{Na} . These results suggest that the increased density of I_{K} is closely linked to the presence of I_{Na} .

In *Xenopus* nerve-muscle cultures a high proportion of the sites of nerve-contact form functional synapses (Anderson *et al.*, 1979) with a localization of post-synaptic specializations (Cohen, 1980; Moody-Corbett *et al.*, 1982). In the present study we did not determine whether each site of nerve-contact, from which we obtained current recordings, had a localization of ACh receptors. However, three cultures were labeled with tetramethylrhodamine-labeled α -bungarotoxin and the proportion of nerve-contacted muscle cells with ACh receptor localization was found to be 60% ($n = 48$ cells), similar to previous studies. Five nerve-contact sites which had a localization of ACh receptors were examined for ionic currents. All five patches of membrane contained I_{K} and four of the membrane patches also contained I_{Na} (Figure 2.5). These results suggest that I_{Na} and/or I_{K} seen at sites of nerve-contact in the muscle cells in the present study were also sites of ACh receptor aggregation.

Figure 2.5. I_{Na} and I_{K} are seen at sites of ACh receptor aggregation. Phase contrast (top) and fluorescence (middle) photomicrographs showing the localization of ACh receptors along the path of nerve-muscle contact in a two day old culture. Bottom: superimposed current traces obtained at the site indicated by an arrow in phase contrast photomicrographs. The membrane potential was stepped to -10 mV and then to +50 mV and I_{Na} and I_{K} recorded, respectively



2.4 Discussion

There are three main findings in the present study: (1) patches of membrane which contain I_{Na} have a high probability of being located near sites of nerve-muscle contact; (2) patches of membrane which contain I_{Na} have a high probability of containing I_{K} ; and (3) I_{K} has a higher density when associated with I_{Na} than in patches of membrane which only contain I_{K} . These results suggest that the voltage-operated ion channels which mediate these macroscopic currents, like the ACh receptor channels, are preferentially localized in a high density at sites of nerve-muscle contact. The data are consistent with recent findings that have demonstrated an increased density of potassium channels at other excitatory synapses (Alonso and Widmer, 1997; Tejedor *et al.*, 1997). Earlier experiments in snake skeletal muscle had failed to reveal an increased density of potassium current near the endplate region (Roberts, 1987). Although this may represent a species difference, it is important to note that the earlier electrophysiological study did not undertake a systematic study of potassium currents and did not attempt to distinguish different types of potassium current (Roberts, 1987). The presence of a higher density of potassium channels at excitatory synapses, as suggested from the present results and from immunohistochemical studies (Alonso and Widmer, 1997; Weldon and Cohen,

1979) would be expected to provide an additional means of regulating post-synaptic excitation and repolarization of the membrane.

This is the first description of an increased I_{Na} density in the region of the nerve contact in *Xenopus* muscle. The findings are consistent with several earlier studies showing increased density of sodium channels at the NMJ of adult vertebrate muscle (Catterall, 1992; Grinnell, 1995). In many adult vertebrate muscle cells the post-synaptic membrane contains deep invaginations with the sodium channels located in the troughs and the ACh receptor channels present at the crests of the fold closest to the pre-synaptic membrane. However, embryonic *Xenopus* muscle in culture, as *in situ*, does not show a highly invaginated post-synaptic membrane (Kullberg *et al.*, 1977; Moody-Corbett *et al.*, 1982; Weldon and Cohen, 1979). Similarly, the post-synaptic membrane of embryonic chick neuromuscular junction is not folded (Frank and Fischbach, 1979; Goodearl *et al.*, 1995) although there is an increased density of sodium channels at these sites (Angelides, 1986). It is not clear what the spatial relationship may be between the ACh receptors and the voltage-regulated ion channels in non-folded membranes. The present experiments were not designed to examine the detailed relationship between sodium, potassium and ACh receptor channels and the exact spatial relationship between these ion channels and other post-synaptic specializations (see below) remains to be determined.

It is unlikely that the increase in current density at sites of nerve-muscle contact were due to channels with an increased single channel conductance being localized specifically at this site. Only one class of sodium channel has been identified in *Xenopus* skeletal muscle (DeCino and Kidokoro, 1985). Our results suggest that these channels are in highest density near sites of nerve-muscle contact. Two classes of outward potassium channels have been identified in *Xenopus* muscle cells in culture (Ernsberger and Spitzer, 1995). The single channel conductance of these two channel classes is similar, close to 20 pS (in 10 mM extracellular potassium), and there is no evidence for potassium channels with a higher conductance. One class of single channel currents shows rapid inactivation, with a time course similar to that of I_{IK} in the present study; whereas the other channel class shows very little inactivation, similar to I_K . These single channel data suggest that an increase in I_{IK} in macro-patches with I_{Na} in the present study, could occur as a result of an increase in the density of channels which mediate I_{IK} .

An estimate can be made of the channel densities of I_{Na} , I_{IK} and I_K from the average current densities, in the macro-patches of the present study. It has been estimated that a sodium current density of 1 mA/cm² corresponds to a density of 20 sodium channels/ μ m² (Caldwell *et al.*, 1986). Based on the average current density recorded at sites of nerve-muscle contact our results suggest a density of 10 sodium channels/ μ m² in these macro-patches. In the

case of potassium channels, the density can be estimated by extrapolating from the single channel conductance reported by Ernsberger and Spitzer (Ernsberger and Spitzer, 1995). From the average current densities reported in this study, our results suggest a density of $10.3 I_{K} \text{ channels}/\mu\text{m}^2$ in macro-patches which contained I_{Na} compared to densities of 3.3 and $3.5 \text{ channels}/\mu\text{m}^2$ in macro-patches which only contained I_{K} or I_{K} , respectively. Taken together, the density of voltage-operated channels (both sodium and inactivating potassium channels) near sites of nerve-contact is $20 \text{ channels}/\mu\text{m}^2$, approximately seven times that found in other areas of the membrane.

The appearance and spatial distribution of ACh receptors is known to be regulated by neuronally derived factors (Grinnell, 1995). Among these, neuronally-secreted agrin plays an important role in the localization of ACh receptors at sites of nerve-muscle contact (Fallon and Hall, 1994; McMahan, 1990) including those found on *Xenopus* muscle cells in culture (Cohen and Godfrey, 1992; Cohen *et al.*, 1995). The neuregulin ARIA (for ACh receptor-inducing activity) has been shown to increase the density of ACh receptors in the membrane (Sandrock *et al.*, 1997; Sandrock *et al.*, 1995). Both agrin and ARIA have also been implicated in the spatial distribution and expression of sodium channels in skeletal muscle. An isoform of a neuronally-derived agrin, which is known to aggregate ACh receptors, is also known to cluster sodium channels in rat skeletal muscle (Sharp and Caldwell, 1996). In addition, Corfas

and Fischbach (Corfas and Fischbach, 1993) have found that ARIA increases the expression of sodium channels in chick skeletal muscle. The results of the present study support the notion that a neuronally-secreted factor regulates the expression and localization of sodium channels in *Xenopus* muscle membrane.

We have found that the density of I_{IK} was increased in membrane patches which contained I_{Na} , compared with membrane patches without I_{Na} . In the absence of I_{Na} , the average density of I_{IK} was similar in membrane patches close to and distant from the nerve contact. The data suggest that it is the co-localization of I_{Na} with I_{IK} which is important in the increased density of I_{IK} , not the direct influence of nerve-contact. These results are consistent with the work of Linsdell and Moody (Linsdell and Moody, 1994; Linsdell and Moody, 1995) who have found that increasing the sodium current density, by expression of foreign sodium channels in the muscle, resulted in an increased density of potassium current in these cells and that blocking sodium channel activity blocked the increase in potassium current density. This work suggests an increase in the density of channels, however it is also possible that the local increase in sodium concentration influences the conductance or activation of individual potassium channels resulting in an increase in potassium current density. Sodium-activated potassium channels have been described in a number of neurons and cardiac tissue (Dryer, 1994). Although the concentration of sodium ions required to activate these potassium channels is apparently high, it may be that the local

environment of the membrane, such as the proximity of sodium and potassium channels and the activity of the sodium-potassium pump, provides the necessary conditions to facilitate activation of potassium channels. Together these studies suggest that membrane excitability, mediated by sodium channel activity, or sodium ion concentration may be important in the regulation of potassium current density.

The architecture of the adult NMJ is highly specialized and includes a number of structural proteins believed to play a role in the localization and maintenance of the ACh receptor channels required for synaptic transmission (Fallon and Hall, 1994; Grinnell, 1995; Sealock and Froehner, 1994). Our results suggest that at least one class of potassium channel should be added to the list of proteins localized at the NMJ. It is interesting to note that at excitatory glutamate synapses, in the central nervous system, and in NMJs of *Drosophila* larva, *Shaker* potassium channels are clustered in the post-synaptic region (Alonso and Widmer, 1997; Gomperts, 1996; Kim *et al.*, 1995; Tejedor *et al.*, 1997). At these synapses, the clustering of *Shaker* potassium channels is believed to be mediated by the membrane-associated guanylate kinase complex which contains PDZ domains (Gomperts, 1996; Sheng, 1996). The PDZ domain preferentially binds to a highly conserved amino acid sequence (S/TXV) which is found in a number of membrane associated or transmembrane proteins, including the C-terminal of the *Shaker* potassium channels (Gomperts, 1996;

Sheng, 1996). In skeletal muscle the syntrophin family of proteins, which are associated with both the sarcolemma and NMJ, also contain the PDZ domain (Adams *et al.*, 1995). The syntrophins bind to the dystrophin associated proteins and at least one member of the syntrophin family, along with utrophin, is known to be restricted to the NMJ (Peters *et al.*, 1997). Gee *et al.*, (1998) have found that this syntrophin associates with the TDVL terminal sequence of sodium channels localized at the junction. The results of the present study raise the possibility that channels mediating I_{K1} may also be associated with this structural protein and play a role in the physiology of post-synaptic specializations in the vertebrate NMJ.

Chapter 3

Cloning and expression of three K⁺ channel
cDNAs from *Xenopus* muscle.

3.1 Introduction

Voltage sensitive potassium channels regulate the frequency, shape and duration of action potentials. A number of studies have described the tightly regulated appearance of voltage gated K^+ currents during the embryonic development of the African clawed frog *Xenopus laevis*. Neurons and muscle show complex developmental programs of ion channel expression which are important for normal maturation. For example, in spinal cord neurons developing in culture differentiation of a non-inactivating voltage activated K^+ current, I_{Kv} , over the first day in culture is required for the transition from a slow primarily Ca^{++} dependent action potential to a fast and predominantly Na^+ dependent one (Barish, 1986; O'Dowd *et al.*, 1988; Spitzer and Ribera, 1998). In these spinal cord neurons maturation of an inactivating K^+ current, I_{KA} , further contributes to the shortening of the action potential and limits the ability of the neurons to undergo repetitive firing (Ribera and Spitzer, 1990; Desarmenien *et al.*, 1993). Skeletal muscle also expresses two large voltage gated K^+ currents during differentiation in culture. One current, I_K , shows little inactivation with membrane depolarization, and the other current, I_{Kf} , shows more rapid inactivation, similar to the I_A (Moody-Corbett and Gilbert, 1992a,b; Spruce and Moody, 1992; Linsdell and Moody, 1995). As the muscle cells mature, both currents are usually expressed (Ribera and Spitzer, 1991; Linsdell and Moody,

1995; Chauhan-Patel and Spruce, 1998; Currie and Moody, 1999), and data from cell attached recordings suggests that I_{K1} and I_K are carried by at least two different structural classes of K^+ channel (Ernsberger and Spitzer, 1995; Chapter 2). The expression of these two channels is not associated with a switch from a Ca^{++} dependent action potential to a Na^+ dependent action potential in skeletal muscle. However, the expression of the K^+ currents is influenced by membrane excitability (Linsdell and Moody, 1994; Spruce and Moody, 1995).

Overexpression of voltage gated Na^+ channels by injection of cRNA caused an increase in the density of both outward currents, and this effect was reduced by treatment with the Na^+ channel blocker tetrodotoxin (Linsdell and Moody, 1994).

In addition, recent experiments in this laboratory have demonstrated that in muscle cells there is a close relationship between I_{Na} and the inactivating K^+ current I_{K1} , relative to sites of nerve-muscle contact (Chapter 2). Taken together, these studies demonstrate the closely associated patterns of expression of the ion channels determining membrane excitability.

Electrophysiology, combined with molecular genetics has revealed the existence of several large multigene families of K^+ channels which shape the unique electrical properties of cells in subtle ways (Jan and Jan, 1997; Hille, 1992). Of particular interest has been the Kv family of voltage gated K^+ channels, which is organized into four subfamilies Kv1, Kv2, Kv3 and Kv4 (Chandy and Gutman, 1993). Thus far homologues of Kv 1.1, Kv1.2, Kv2.1,

Kv2.2 and Kv3.1 (Ribera, 1990; Ribera and Nguyen, 1993; Burger and Ribera, 1996; Gurantz *et al.*, 2000), and Kv4.3 (Lautermilch and Spitzer, unpublished clone accession # U89265) have been isolated from *Xenopus* and the differential expression of some of these genes has been examined in spinal cord neurons. However, to date the molecular identities of the channels responsible for the observed inactivating and non-inactivating current in *Xenopus* skeletal muscle are unknown. The purpose of this work was to determine the molecular identities of the K⁺ currents in *Xenopus* muscle which may underlie each of the observed outward K⁺ currents. We report here the molecular cloning of three Kv1 K⁺ channel α -subunits from *Xenopus* muscle, describe the current phenotype produced by each in HEK 293 cells and describe their developmental expression.

3.2 MATERIALS AND METHODS

3.2.1 Muscle Isolation and first strand cDNA synthesis

The muscle used for the isolation of cDNA clones was obtained in one of three ways: (1) muscle cells were isolated from stage 19-22 embryos and used directly, (2) muscle cells were isolated from stage 19-20 embryos and grown in culture for three days, or (3) sartorius muscle was isolated from adult frogs. All procedures were in accordance with Canadian Council of Animal Care

guidelines, and animal care procedures of Memorial University of Newfoundland.

Embryos were staged according to Nieuwkoop and Faber (1967) and placed in dissecting medium containing 2/3 Leibovitz L15 medium, 0.5% heat inactivated horse serum, 0.01 mg/ml gentamycin, and 5 units/ml nystatin (all reagents from Gibco-BRL, Burlington ON). Under sterile conditions and using insect pins the back portion of the embryo was dissected away from the body and placed in fresh dissecting medium containing 0.5 - 1 mg/ml collagenase (Gibco-BRL, Burlington ON) for 10 - 20 min. The myotomal muscle layer was dissected away from the notocord, neural tube and skin. In some procedures muscle obtained from stage 19-22 embryos was used directly for extracting RNA. In other procedures muscle cells from stage 19-20 embryos were isolated in a similar manner and then placed in culture as previously described (Anderson *et al.*, 1977). Only cultures that were visibly free of neuronal cells were used.

An adult frog was anesthetized in 2 mg/ml tricaine solution for 20 min, placed on ice and decapitated. The sartorius muscle was dissected free and placed in frog Ringers solution (111 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.2).

Total RNA was extracted using TRIZOL reagent (Gibco BRL, Burlington, ON), and treated with RQ-1 Dnase (Promega, Madison, WI) to remove any contaminating genomic DNA. Typically, reverse transcription (RT) was carried

out using 1-3 μ g total RNA in a 40 μ l reaction, however in the case of the muscle cultures, approximately 100 ng of RNA was used in a 20 μ l reaction. The RT reaction was performed using MMLV reverse transcriptase (Gibco BRL, Burlington, ON) and one of three types of primers: oligo dT primer, random hexamers (Amersham Pharmacia Biotech, Baie d'Urfe, PQ) or gene specific primer (listed in Table 1 and discussed below).

3.2.2 Isolation of specific K⁺ channel cDNA sequences

As described in the Results section, three K⁺ channel cDNAs were isolated, corresponding to Kv1.2 and Kv1.4 isoforms and a novel K⁺ channel we have called XKv1.10. The Kv1.2 cDNA was isolated using RT-polymerase chain reaction (PCR) and the Kv1.4 and XKv1.10 cDNAs were isolated using RT-PCR and rapid amplification of cDNA ends (RACE) PCR (Frohman, 1989).

Preliminary data (not shown) suggested that Kv1.2 mRNA was present in *Xenopus* myocytes in culture. We designed PCR primers (Table 3.1) to the 5' (2-A) and the 3' (2-B) ends of the *Xenopus* Kv1.2 channel cDNA (Ribera, 1990) and amplified K⁺ channel cDNA from the cultured muscle cell cDNA. To amplify other Kv1 K⁺ channels which may be present in *Xenopus* muscle we designed two Kv1 specific PCR primers. One of the primers, Deg 1, was designed against cDNA encoding the T1 tetramerization domain (Li *et al.*, 1992; Shen *et al.*, 1993), and the second primer, H5, was designed against cDNA encoding the highly conserved signature sequence of the H5 pore region

(Heginbotham *et al.*, 1994; Table 3.1). RT-PCR was carried out with Deg1 and H5 using 1 μ l of cDNA derived from adult sartorius muscle as template. Two PCR products were generated: a 1 kb product, and a 1.1 kb product. The 5' and 3' ends of these two cDNAs were isolated using RACE PCR and a series of internal primers. The XKv1.4-3' RACE PCR was carried out using the 4-G primer and the oligo dT primer. The XKv1.10-3' RACE PCR was carried out using 10-C and the oligo dT primer. The XKv1.4-5' RACE reaction was carried out using 4-B, 4-C, 4-D, 4-E and 4-F and oligo dT primers, while the XKv1.10-5' RACE reaction was carried out with the 10-A, 10-B and oligo dT primers.

The template cDNA for isolating Kv1.4 was sartorius muscle and the template cDNA for isolating Kv1.10 was embryonic myotomal muscle. The template for XKv1.4-3' RACE reactions was oligo dT primed sartorius muscle cDNA, while the template for XKv1.4-5' RACE was either 4-A primed or random primed sartorius muscle cDNA. The template for XKv1.10-3' RACE reactions was oligo dT primed embryonic myotomal muscle cDNA, while the template for XKv1.10-5' RACE was random primed embryonic myotomal muscle cDNA. The cDNA template for 5' RACE was prepared and tailed with poly dA as previously described (Frohman, 1989).

To confirm the identity of the full length Kv1.4 and Kv1.10 RACE products, we amplified the entire coding regions utilizing primers designed against selected 5' and 3' untranslated cDNA. These PCR products were then

Table 1. Primers used for XKv1.2', XKv1.4 and XKv1.10 RT-PCR and RACE reactions. Numbers given in the nucleotides column indicate primer position in the appropriate cDNA: XKv1.2' (Figure 3.1A), XKv1.4 (Figure 3.2A) and XKv1.10 (Figure 3.3). Oligo dT primer was used in 5' and 3' RACE for XKv1.4 and XKv1.10, and thus nucleotide position is not applicable (N/A).

K ⁺ Channel	Primer Name	Direction	Sequence	Nucleotides
<u>XKv1.2</u>				
	2-A	upstream	ATGACCGTTGCAACCGGAGATCTG	1-24
	2-B	downstream	TTAAACATCAGTTAGCATTTTGGTTA	1473-1500
	2-C	upstream	TTTTGCCCTGAAACTTTACC	592-611
	2-D	downstream	GGAGTCCAAATTCCTCATGCTGGC	1021-1045
<u>XKv1.4</u>				
	H5	downstream	ACTGTTGTCATGGAACTACAGCCCACCA	2389-2417
Deg 1		upstream	TCNGGNITGCGCCTTIGARACSC	1324-1344
	4-A	downstream	AGACTCTTTGAGCCCTTCCTCC	2685-2706
	4-B	downstream	GTGCGATAGTCAATACCCCTGC	2482-2503
	4-C	downstream	GTGTGACCAAGTATCTGCAGCC	2231-2252
	4-D	downstream	TCTCATAGCGCATTCTGCACCG	1320-1342
	4-E	downstream	CCGACTGGTAGTAGTATAGG	1470-1490
	4-F	downstream	ATATCCGTAAGGCAGGTGGTTA	814-835
	4-G	upstream	CCTAGTAACAGGGCAACAATTCC	2103-2126
	4-H	upstream	CATGGAGGTTGCAATGGTGAGTG	760-783
	4-I	downstream	GTTCTCTTTCATGGTTTCTGC	3119-3140
	4-J	upstream	GGTACAGTCCACTGAGCAGTCTC	672-694
	4-K	upstream	CAGCAGCAGAGATCCACCTTG	751-769
	4-L	downstream	ATATATTTACACATCAGTTTCCAG	2783-2800
	Oligo dT	N/A	GACATCGATTTTTTTTTTTTTT	N/A
<u>XKv1.10</u>				
	H5	downstream	ACTGTTGTCATGGAACTACAGCCCACCA	1450-1478
Deg 1		upstream	TCNGGNITGCGCCTTIGARACSC	489-512
	10-A	downstream	TCCCGGAATTTTCTGATTACATCC	732-755
	10-B	downstream	GTTAGCTCGATAAGGATGTACCGG	961-984
	10-C	upstream	TAACAGCGCACACCATTTTAAGG	981-1003
	10-D	upstream	ACAACACTGAGAATACCAATATGCC	344-368
	10-E	downstream	GAGACAGTATTAAGTGAAGACATTCC	1838-1863
	Oligo dT	N/A	GACATCGATTTTTTTTTTTTTT	N/A

cloned and sequenced as described below.

3.2.3 PCR Conditions and Programs

All PCR for isolation of K⁺ channel cDNA was carried out in volumes of 50 μ l. Each reaction contained 1X PCR buffer (20 mM Tris pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 1.25 units platinum Taq polymerase (Gibco BRL Burlington, ON), 0.2 mM dNTPs and 0.5 μ M of each primer.

Reactions were performed using a Hybaid PCR Express thermocycler (Franklin, MA). For RT-PCR and 3' RACE, the thermocycler was set for one cycle at 94° C for 4 min; 30 cycles at 57° C for 1.5 min, 72° C for 1.5 min, 94° C for 1 min; and one cycle at 57° C for 1.5 min, 72° C for 10 min. For 5' RACE, the thermocycler was set for one cycle at 94° C for 4 min, 57° C for 1.5 min, 72° C for 10-20 min, 94° C for 1 min; 30 cycles at 57° C for 1.5 min, 72° C for 1.5 -2 min, 94° C for 1 min; and one cycle at 57° C for 1.5 min, 72° C for 10 min (Frohman, 1989).

PCR products were electrophoresed on 1 - 1.25% agarose gels containing 0.5 μ g/ml ethidium bromide, and visualized under UV light. The images were digitized with an AlphaImnotech ChemiImager 4000 (San Leandro, CA). Figures were prepared with Adobe Photoshop V5.5 (San Jose, CA).

PCR products were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). Nucleotide sequences were determined using the T7 sequenase kit (Amersham Pharmacia, Baie d'Urfe, PQ), or alternatively, with an automated

sequencer at the Centre for Genomics, Hospital for Sick Children (Toronto, ON, Canada). DNA sequences were submitted to the BLAST server (www.ncbi.nlm.nih.gov/BLAST) for database and pairwise comparisons (Altschul *et al.*, 1997).

Phylogenetic analysis was carried out on the proteins predicted to be encoded by the K⁺ channel cDNAs isolated here and the other known *Xenopus* Kv channel cDNAs using Clustalw (Thompson *et al.*, 1994) MEGA2 (Kumar *et al.*, 1994) phylogenetic tree construction software.

RT reactions with no reverse transcriptase enzyme (RT-minus) were carried out in parallel with all RT reactions and tested by RT-PCR to ensure there was no contaminating genomic cDNA.

3.2.4 Recording K⁺ currents from HEK 293 cells transiently transfected with expression vectors encoding XKv1.2', XKv1.4 and XKv1.10.

Plasmids and DNA constructs

The open reading frame of XKv1.2' was amplified by RT-PCR from embryonic muscle cDNA, cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. The XKv1.2' insert was excised from the pCR2.1-XKv1.2' (PCR amplification and cloning described above) construct with Eco R1 (Gibco BRL, Burlington, ON), and the XKv1.2' insert was then ligated into Eco R1-digested pCDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, CA). The open reading frame of XKv1.4 was amplified by RT-PCR from embryonic muscle

cDNA with primers 4-K and 4-L. The PCR conditions and program were as above, with 60 °C annealing temperature, and extension for 2.5 minutes. The PCR product was cloned into pCR2.1, and sequenced. The XKv1.4 insert was excised from the construct with Eco R1, and ligated into Eco R1-digested pCDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, CA). The open reading frame of XKv1.10 was amplified by RT-PCR with primers 10-D and 10-E using stage 21 muscle cDNA. The PCR conditions and program were as for XKv1.4. The PCR product was cloned into pCR2.1 and sequenced. The XKv1.10 insert was excised from the construct with Bam H1 and Not 1 (Gibco BRL, Burlington, ON), and ligated into Bam H1 and Not 1-digested pCDNA3 eukaryotic expression vector (Invitrogen, Carlsbad, CA). Restriction mapping and DNA sequencing confirmed the identity of the constructs.

Cell culture and transfections

HEK 293 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained at 37 °C, 5% CO₂, in Dulbecco's minimal essential media (DMEM, Gibco BRL, Burlington, ON) supplemented with 10% fetal calf serum, penicillin (10 µg/ml) and streptomycin (10 units/µl). Briefly, cells were plated on 12 well plastic culture dishes and allowed to grow to 70% - 90% confluency. Transient transfections were carried out using 5 µg LipofectAMINE 2000 (Gibco BRL, Burlington, ON), 2 µg of one of the three K⁺ channel pCDNA3 constructs, and 0.5 µg of a green fluorescent protein (GFP) construct (Clontech,

Washington, DC; for identification of successfully transfected cells) diluted in 500 μ l DMEM. The cells were allowed to recover for 5h, removed by gentle titration or trypsinization and re-plated at lower density onto collagen coated glass coverslips in culture chambers.

Electrophysiological recording

The functional expression of the XKv1.2', XKv1.4 and XKv1.10 constructs in HEK 293 cells was assessed by whole cell patch clamp recording (Hamill *et al.*, 1981) 24-48 hours after transfection. Whole cell current recordings were made using a List EPC-7 patch clamp amplifier (Medical Systems Corp, Greenvale, NY). Cells were examined on a Zeiss IM 35 equipped with fluorescence optics. Only green fluorescing cells that possessed simple morphology and not in contact with other cells were used for electrophysiology. The extracellular recording solution in the bath contained (in mM): 143 NaCl, 2 KCl, 1.2 CaCl₂, 1 MgCl₂, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4 adjusted with NaOH). The patch electrode contained (in mM): 140 KCl, 1 1,2 bis [2 aminophenoxy]-ethane-N,N,N',N'-tetraacetic acid (BAPTA), 5 MgCl₂, and 10 HEPES (pH 7.4 adjusted with NaOH). Pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) using a PP-83 two stage vertical puller (Narashige, Japan) and had resistances of 10-13 M Ω when filled with the patch pipette solution. pClamp software (version 6.0.1, Axon Instruments, Foster City, CA) was used for voltage control, data acquisition and

analysis.

During an experiment, the cells were held at -80 mV and depolarizing steps were applied in 10 mV increments every 10 s. The data were digitized at 10 kHz and filtered at the amplifier at 3kHz. Current density measurements were obtained by dividing current by cell capacitance (obtained from the capacitance dial on the amplifier). Peak outward current was determined during the 100 ms depolarization step to 30 mV, mean current was determined from the mean current between 75 to 85 ms after depolarization to 30 mV. Currents were not leak subtracted.

3.2.5 Analysis of developmental and tissue specific expression of XKv1.2', XKv1.4 and XKv1.10 mRNA

Developmental expression of the three K⁺ channel RNAs was examined using RT-PCR in embryos from stage 2 (cleavage), 8 (blastula), 10.5 (early gastrula) 11.5 (mid gastrula), 12.5 (late gastrula), 14 (early neurula), 17 (mid neurula), and 21 (tail bud), and tadpoles from stage 31 (early tadpole) and 50 (late tadpole). Total RNA was extracted from two whole embryos or tadpoles, treated with RQ-1 Dnase (Promega, Madison WI), phenol extracted, ethanol precipitated, and resuspended in 10 μ l DEPC H₂O. 1.5 μ l of this RNA was used for template in a 40 μ l RT reaction with random primers. These RT products were used as template for PCR to detect K⁺ channel and molecular marker transcripts with the following primer sets: to detect XKv1.2' we used 2-C/2-D

which amplified a 300 bp product; to detect XKv1.4 we used 4-G/4-C which amplified a 400 bp product; to detect XKv1.10 we used 10-B/10-D which produced a 600 bp product; and to detect H4 histone we used H4-A/H4-B which produced a 250 bp product .

The RT-PCR conditions were as described above. For all of these reactions the annealing temperature was 60° C except for the XKv1.2' primer set 2-C/2-D where the annealing temperature was 57° C. Furthermore, the number of amplification cycles varied depending on abundance of the transcript. XKv1.2' was amplified for 29 cycles, the XKv1.4 and XKv1.10 were amplified for 27 cycles, and the molecular marker H4 was amplified for 22 cycles. The thermocycler was set for one cycle at 94° C for 4 min; 22, 27 or 29 cycles (as indicated above) at 57° C (XKv1.2) or 60° C (all others) for 1 min, 72° C for 1 min, 94° C for 1 min; and one cycle at 57° C or 60° C for 1 min, 72° C for 10 min. All reactions used an amount of RT template which yielded an equivalent amount of the H4 histone PCR product.

The expression of three K⁺ channel RNAs was also analyzed in muscle cells isolated from stage 14 embryos (n =10), stage 21 embryos (n = 10) and adult sartorius muscle (one adult), as well as spinal cord from stage 21 embryos (n =10). The total RNA extracted from the embryonic tissue and 100 ng of RNA from the sartorius muscle was reverse transcribed in 40 µl reactions (as described above). One µl of these RT products were used as template for RT-

PCR.

The RT-PCR conditions and amplification programs were as described for the whole embryo above, except that more amplification cycles were required due to a lower concentration of cDNA template. The XKv1.2' was amplified for 31 cycles, the XKv1.4, XKv1.10 were amplified for 29 cycles, and the molecular marker H4 was amplified for 24 cycles. An additional primer set was used for RT-PCR to amplify the neuronal molecular marker medium weight neurofilament (NFM-A/NFM-B). The number of cycles and annealing temperature for this marker were identical to those for XKv1.4 and XKv1.10. Both reactions were carried out a total of three times with two separate samples of cDNA.

3.3 Results

3.3.1 Isolation and sequence analysis of three K⁺ channel cDNAs: XKv1.2', XKv1.4 and XKv1.10

XKv1.2' isolated from muscle is nearly identical to XKv1.2

The cDNA for XKv1.2' possessed an open reading frame of 1497 bp (Figure 3.1A), and was 95% identical to the XKv1.2 cDNA reported by Ribera (Ribera, 1990). The predicted amino acid sequence of our clone shared 98% identity with that of the XKv1.2 protein. This clone was designated XKv1.2'. The most significant divergence between the predicted amino acid sequence of the

Figure 3.1 Analysis of the XKv1.2' cDNA A. Nucleotide sequence of the XKv1.2' cDNA isolated from embryonic muscle, and the deduced amino acid sequence. The position of the nucleotide is indicated on the left, and amino acid on the right. B. Alignment of the deduced amino acid sequence from XKv1.2' with XKv1.2 and mouse Kv1.2 (mKv1.2). Identical and similar residues are shaded black. The position of the tetramerization domain (T1), membrane spanning domains (S1-S6) and pore domains are indicated with a dashed line. The six amino acids (11-16) near the N-terminus differing from XKv1.2 (Ribera, 1990) are indicated. Putative N-linked glycosylation site in the S1-S2 linker and phosphorylation sites by protein kinase C (PKC), protein kinase A (PKA) and tyrosine kinase (Tyr-K) are also indicated.

1 M T V A T G D L T D E A S A L P G H P Q D S Y D P E P D H E 30
 A T G A C C G T T G C A A C G G A G A T C T G A C A G T G A A G C G T G C G C T T G C C A G G T C A C C A C A G G A C A G T T A T G A C C T G A A C C A G A T C A T G A G
 91 C C E R V V I N I S G L R F E T Q L K T L S Q F P E T L L G 60
 T G T T G C G A A G A G T G G T T A T A T A T T C A G G A C T G C G G T T T G A A C G C A G C T G A A A C C T T G T C A G T T C C C T G A A A C T T A C T G C G G
 181 D P K K R M R Y F D P L R N E Y F F D R N R P S F D A I L Y 90
 G A T C T A A A A A A G A T G A G A T A C T T T G A T C C C T T G A G A A T G A G T A T T T T T G A C A G A A C A G G C C A G T T T T G A T G C T A C T T A T A C
 271 F Y Q S G G R L R R P V N V P L D I F S E E I R F Y E L G E 120
 T T T T A C C A G C T G G T G G C A G A T A A G G A G A C T G T A A T G T G C C T T G G A T A T A T T T C A G A A G A A T C C G G T T C A T G A A C T T G G T G A A
 361 E A M E I F R E D E G F I K E E E R P L P D N E F Q K Q V W 150
 G A A G C C A T G G A A A T C T C C G G A A G A T G A A G A T T T A T A A G A A G A A A C G C C C T T G C C A G A C A A T G A A T T C A A A A C A A G T G T G G
 451 L L F E Y P E S S G P A R I I A I S V T I L I S I V S F 180
 C T C T T G T T G A T A C C T G A G A G C T C G G G A C A G C A G G A T T A T A G C A T A C A T C A G T T A C G G T A T A T A A T A A T C A A T G T C A G C T T
 541 C L E T L P V F R D E N E D M H G S G G N Y Y S Y P N S T V 210
 T G C C T T G A A A C T T T A C C A G T T T T C A G A G A T G A A A A T G A A G A C A T G C A C G G A A G T G G A G A A A T T A C T A C T C A T A C C C C A A T A G C A C A G T G
 631 R F Q K S N T F T D P F F I V E T L C I I W F S F E F L V R 240
 C G A T T T C A A A A A T C A A A T A C T T T T C A G A T C C T T C T C A T T G T G A G A C T C T G T G C A T C A T C T G G T T T C T T T G A G T T C T G G T T C G T
 721 F L A C P S K A V F F T N L M N I I D I V A I I P Y F I T L 270
 T T C C T G C G C T G C C C A A G T A A G C T G T C T T T T T A C C A A C C T C A T G A A C A T C A T G A T A T A G T T G C A A T C A C C C T T A C T T T A T T A C A T T A
 811 G T E L A E K T E D G Q Q G Q Q A M S L A I L R V I R L V R 300
 G G C A C G G A G C T G G C A G A A A A A C T G A A G A T G G G C A C A G G G C A G C A A G C A A T G T C T T T A G C A A T C T T A G A G T T A T C A G A C T G G T T A G A
 901 V F R I F K L S R H S K G L Q I L G Q T L K A S M R E L G L 330
 G T A T T T A G G A T C T T A A G C T C T C T A G A C A T T C A A A G G G T C A C A A A T C T T G G G C A G A C T C T G A A A G C C A G C A T G A G G G A A T T G G G A C T C
 991 L I F F L F I G V I L F S S A V F F A E A D E R D S Q F P S 360
 C T A A T A T T T T T C T C T T A T T G G C G T T A T A C T C T T T C T A G T G C A G T G T T T T T G C T G A A G C T G A T G A G A G A G A T T C C A G T T T C C A A C
 1081 I P D A F W W A V V S M T T V G Y G D M V P T T I G G K I V 390
 A T C C A G A T G C C T T C G T G G G C T G T A G T T C C A T G A C A C A G T A G G C T A T G G A G A C A T G G T C C C T A C A C A A T A G T G G C A G A A A T A G T A
 1171 G S L C A I A G V L T I A L P V P V I V S N F N Y F Y H R E 420
 G G C T C T C T G T G C A A T T G C A G G C G T A T A C C A T T G C C T A C C T G C C C T G T T A T A G T A T C C A A T T C A A T T A C T T C T A T C A T A G A G A G
 1261 T E G G E Q A Q Y L Q V T S C P K I P S S P D L Q K S R S A 450
 A C A G A G G G G G G A G C A A G C A A A T A T C G C A A G T A A C C A G C T G T C C A A A G A T C C C T T C A T C C C C T G A C C T T C A A A A G A G C A A G A T G C C
 1351 S T L S K S D Y M E I Q E G V N H S N E D F R E K N L K T A 480
 T C C A C T C T G A T A A T C G G A C T A T G A A A T T C A G A A G G G T A A A T C A T A G C A A T G A A G A T T T T A G A G A G A A A C T T A A A A C T G C A
 1441 N C T L G N T N Y V N I T K M L T D V 499
 A A T T G C A C A T T A G A A A C A A A A T T G T T A A T A A C A A A A T G C T A A C T A G T G T T T A A G

----- Tyr K T1

XKv1.2' : MTVATGDLTDASALPGHPQDSYDFEPDHECCERVVINISGLRFETQLKTLSSQFPETLLGDPKKRMRYFDPLRNEYFFDRN 81
 Xkv1.2 : MTVATGDLTDGSGVGAHPQDSYDFEPDHECCERVVINISGLRFETQLKTLSSQFPETLLGDPKKRMRYFDPLRNEYFFDRN 81
 mKv1.2 : MTVATGDPVDRRAALPGHPQDTYDFEADHECCERVVINISGLRFETQLKTLSSQFPETLLGDPKKRMRYFDPLRNEYFFDRN 81

XKv1.2' : RPSFDAILYFYQSGGRLRRPVNVPLDIFSEIRFYELGEEAMEIFREDECFIKEERPLPNEFQKQVWLLFEYPSSGPA 162
 Xkv1.2 : RPSFDAILYFYQSGGRLRRPVNVPLDIFSEIRFYELGEEAMEIFREDECFIKEERPLPNEFQKQVWLLFEYPSSGPA 162
 mKv1.2 : RPSFDAILYFYQSGGRLRRPVNVPLDIFSEIRFYELGEEAMEIFREDECFIKEERPLPNEFQKQVWLLFEYPSSGPA 162

S1 N-Gly S2

XKv1.2' : RIITAIISVIVILISIVSFCLETLPVFRDENEDMHGSGNNISYFNSTVRFQKSNITFDFFIVETLCIIWFSFEFLVRFLA 243
 Xkv1.2 : RIITAIISVIVILISIVSFCLETLPVFRDENEDMHGSGNNISYFNSTVRFQKSNITFDFFIVETLCIIWFSFEFLVRFLA 243
 mKv1.2 : RIITAIISVIVILISIVSFCLETLPVFRDENEDMHGSGNNISYFNSTVRFQKSNITFDFFIVETLCIIWFSFEFLVRFLA 243

S3 S4 PKC *

XKv1.2' : CPSKAWFTTNLMHIIDIVAIIPYFITLGTLEAETEDQOQOQAMSLAILRVIRLVRFRIKLSRHSKGLQILQOTINAS 324
 Xkv1.2 : CPSKAWFTTNLMHIIDIVAIIPYFITLGTLEAETEDQOQOQAMSLAILRVIRLVRFRIKLSRHSKGLQILQOTINAS 324
 mKv1.2 : CPSKAWFTTNLMHIIDIVAIIPYFITLGTLEAETEDQOQOQAMSLAILRVIRLVRFRIKLSRHSKGLQILQOTINAS 324

S5 PORE S6

XKv1.2' : MRELGLLIFFLFIGVILFSSAVVFAEADERDSQFPSIPDAFWAVVSMITTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALF 405
 Xkv1.2 : MRELGLLIFFLFIGVILFSSAVVFAEADERDSQFPSIPDAFWAVVSMITTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALF 405
 mKv1.2 : MRELGLLIFFLFIGVILFSSAVVFAEADERDSQFPSIPDAFWAVVSMITTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALF 405

Tyr K PKA Tyr K

XKv1.2' : VPIVSNFNFIYHRETEGQEQAYQLQVTSCKPIPSFDLQKRSASTLSKSDYMEIQEGVNHSDNEDFREKSLKTANCTLON 486
 Xkv1.2 : VPIVSNFNFIYHRETEGQEQAYQLQVTSCKPIPSFDLQKRSASTLSKSDYMEIQEGVNHSDNEDFREKSLKTANCTLON 486
 mKv1.2 : VPIVSNFNFIYHRETEGQEQAYQLQVTSCKPIPSFDLQKRSASTLSKSDYMEIQEGVNHSDNEDFREKSLKTANCTLON 486

XKv1.2' : TNYVNIITQGLTDV : 499
 Xkv1.2 : TNYVNIITQGLTDV : 499
 mKv1.2 : TNYVNIITQGLTDV : 499

XKv1.2' and XKv1.2 was a change in a stretch of six amino acids (11-16) near the amino terminus where the XKv1.2' cDNA moves out of, then back into reading frame with respect to XKv1.2. Five of these six amino acids in the predicted XKv1.2' protein were identical to the corresponding amino acids in mKv1.2, but none were identical to the corresponding amino acids in the previously cloned XKv1.2 (Figure 3.1B). There were several nucleotide changes that caused no change in amino acid sequence as well as two other nucleotide changes that caused two non-conservative amino acid changes, one in the S4-S5 linker, and the other in the intracellular C-terminal domain. As observed with other Kv1 proteins, there were multiple potential sites for post-translational modification in the XKv1.2' protein (Figure 3.1B). The S1-S2 extracellular loop contained a potential site for N-linked glycosylation and there were multiple potential sites for phosphorylation, including a potential site for protein kinase C (PKC) in the S4-S5 linker, a potential site for protein kinase A (PKA) in the C-terminal about 30 amino acids from the S6 domain, and three potential sites for tyrosine kinase phosphorylation in the C-terminal region. These results suggest that XKv1.2' is expressed in *Xenopus* muscle as well as in spinal cord neurons as reported by Ribera (1990).

XKv1.4 is structurally similar to rapidly inactivating K⁺ channels

Five overlapping clones obtained by RT-PCR, 3' and 5' RACE ultimately produced 3273 bp of cDNA (Figure 3.2A). RT-PCR with primers 4-I and 4-J

Figure 3.2 Analysis of the XKv1.4 cDNA A Nucleotide sequence of the XKv1.4 cDNA isolated from muscle, and the deduced amino acid sequence. The position of the nucleotide is indicated on the left, and amino acid on the right. Potential MyoD binding sites (CANNTG) in the 5' UTR are shaded in gray. B. Alignment of deduced amino sequence from XKv1.4 with mouse Kv1.4 (mKv1.4), chick (cKv1.4) Kv1.4 and the *Shaker B* channel. Identical and similar residues are shaded in black. The position of the ball domain, tetramerization domain (T1), membrane spanning domains (S1-S6) and pore domains are indicated. Putative N-linked glycosylation site in the S1-S2 linker and phosphorylation sites by protein kinase C (PKC), protein kinase A (PKA), calcium/calmodulin dependent protein kinase (CaMKII) and tyrosine kinase (Tyr-K) are indicated.

1 GGTGTTACATCCCAAGGCGCTTCCAAGCGGATTTTTAAAAAACAACAAATGTTATTGGACAGTGCTTTATGAAACTGCTGCGCATG
 91 GGAGTTTGCGCATGTTTGGCTTCTAGGACAAATACCTGAAGAGATATCTGGCTGGCTTTGAGTGAACCTTTTACAGCTTTTTC
 181 CACACAGATTTTGGCTTTAGAGAGCTCTAGGACATCTTGGACATCTCTCTCTGCAAAAAAATCTGGAGAACTTCTGTCAC
 271 CCCTCTGCAAAATGTTGGTCCACCTGCGCCAGAGCTCACTGGCTCTCTGTTCAATTTTCTCTCCAGAGGCTCTCACTTTAAAGGCGCGA
 361 AAATACCACTGAGACAGAGTCCGAGCTGGCAATCTGTGGGTTCTGGCAAAAGCCGAGGGGCTCGTGAATAGGTCATAGACACTCA
 451 TTTAGTGGGCCAAGTGGGGTGTGTTTGGCCACCCTGTGGGCTGTGGAGGCTCTGGGATCAAGATGCCCGGGGCTATTGTTGAGTCTCA
 541 GTTCAGACCTGCTTGGAGCACTGAGGCGCTTGGCTTCAATTTGTTTGTCTGACAGATTCAGCTGAAGTCAAAAGGCGAAGTGGCGGCG
 631 ACTGAGAGGCAAAAACACTGGATTAAGATTTTATTGCGACCATGACAGGGTGGTACAGTCCACTGAGCAGTCTCCCTGTGACAGAGTAA

 721 TAGTGAGCTCACGGGAACGCGACCTCCGACGACGAGATGGAGGTTGTCATGGTGAAGTGGGACCTTCGGGCTGTAGCAACCACTG

 811 FYGYAQARARERERQAHSRATAAAAASGEG
 CTTTACGATATGACAGAGGCCGCGCCGAGCGGAACGCCAGGCGCATCCCGGCCACAGCGCTGCTCTGAGGAGAGGC

 901 GNSGGGAGGVVNARRAPQNVQVPERQEEKSSQK
 GGAACCTCAGAGGGCGGAGCGGAGTGAACGCGCGCGCGGCTCAGAAATCAAGTGCACAGAGCGGAGAGGAGAGATCATCGCAGAAA

 991 KKSACRRRRYVWFLSGGNCNRWRWSRHNHCCSGGGAGG
 AAGAAAAGTCAGGCGGAGTACTGGCCATTAGCGGCTGCAACAGGTGGAGGCGGCGCAACGAATGACGAGCGGCTGGAGGA

 1081 RRAAGAGCGEDDDGTTFFSELGLGCGSEEMMLREEVA
 AGAAGAGCAGGAGGAGAGACGACGCGCACTTCCCTCGGAGCTGGGTTCTATGGGCTCTGAGGAGATGATGCTCAGGAGAGAGTGGCT

 1171 EEDQKFYIEICEDEKEANSLHRRSPTEDEGY
 GAGGAGACCAAAAGTTTACATTTGTGAAGAGGATGAGAAGGAGGCCAACAGCTGACAGGAGGAGAGAGCCACAGAGGATGAGATAT

 1261 HPVYSEFECECERVVINVSGMRYETQLKTL
 CACCCTGTGACAGCGAGTTGAGTGTCTGAGAGGGTGTGATCAAGTGTGAGGATGGCTGATGAGACCGCATTAAGAACTTTGAGC

 1351 QFPETLLGDPEKRRTRYFDPLRNEYFFDRNR
 CAGTTTCCCGAAACTCTGCTGGGCGACCCAGAAAAAGGACGCGCTATTGCAAGCTCTGAGGAGACGAGTATGTTCTGCGCAACCGCG

 1441 LSFIDSYLVSQSGRLKRPVNVFPDIFSEE
 CTGAGTTTGCATCCATCTATACCTACAGTCCGCGGAGGCTGAAGCGGCGGCTCAAGTGGCTCTGCATCTTTTCCGAGGAG

 1531 VKFYELGEEALLKYREDEGFVKEEEKQLPE
 GTCAGTTTACGAATTTGGGAGAGGAGGCTCTAAAAACCCGAGGATGAAGTTTCGTGAAGAGGAGAGAGAGCCAGCTGCCAGAA

 1621 NEFKKQVWLLFEYPESSGAARGIAIVSVLV
 AATGAGTTCAAGAGCAAGTGTGGCTACTGTTTGAATACCGGAAAGTTCGGGGGCGAGCTCGGGGCTATGCCATGCTCTGAGTGTGGTC

 1711 ILISIVIFCFLETLTPEFRDDDKNLLSLFLGLMGH
 ATCTCACTCCATTTGTCATCTTTTGTGAACATTAACGAGTTCAGGATGACAGGATATTTGCTGCTCTCCGCTGGGAATGGGG

 1801 DDDGAGEDGEDGEGGAYNATFLSTSDSGHTAFND
 GATGATGATGTTGAGGGAGATGAGAGGAGGGGCTTCAATGCAACTTTCTATCAACAGAGATGGTCACTGATCTTAATGAT

 1891 PFFIVETVCIVWFSPFEFAVRLFACPKPEF
 CCATTTTCTAGTGGAGCTGTGTGCTATGCTGGTCTCTCTGAGTTTGTGTGCGGCTTTTGTCTGCGCGAGCAACCTGAATTT

 1981 FKNIMNIADIVASILPYFITLTGTEFGQHP
 TTTAAAAATAATGAACATAATGAGCATTTGTGCTATTTGCTTACTTTATCAAGCTGGGTACTGAGCTGGGACGAGCAACCGGCT

 2071 QQQQHLALVTGQQLPQGTGQQQQQAMSFAL
 CAGCAGCAGCAGCTTGGCTAGTAAACAGGGGCAACACTTCCCGAGGAACTGGGCGAGCAACAGCAGGCTATGCTTTGCTATCTG

 2161 RIIRLVFRIFKLSRHSKGLILGLTLRA
 AGGATAATTCGCTGGTGAAGGCTTCCGAATCTTAAATGTCCAGGCAATTCAGAGGGCTGAGATCTGCTGACACCTTGAGAGCC

 2251 SMRELGLLLILFLFIGVILFSSAVYFAEAD
 AGCATGAGGGAACCTGGGATTAATCTCTCTTTTCTGAGGCTATCTAATCTCAAGTGTGTTTATTTTTCAGAGGCGGATGAA

 2341 PTTTFQSIPTDAFWNAVVTMTTGTGVTGDMKPT
 CCGACACCACTCTCAGAGCATCTGATGCTCTGTTGGGCTGTGTTTCAATGACACAGTGGGTATGCGGATATGAGGACCACT

 2431 TVGSKIVGSLIAGVLTIALPVPVIVSNF
 ACTGTTGGGGTAGATAGTGGTTCCTGTGTGSCATAGCAGGGGTATTGATATCGCATACCACTGAGTGCAGTATGATGTTTCAAATTT

 2521 NYFYHRETDNNEEQTLTQSSSSCPYLPNTNL
 AACTACTTTTACATAGGGAATGATAATGAAGACAAACACAGTATAACACAGCAGCTCCAGTGGCATCTTACCAAGCAACCTA

 2611 LKKLRSSTSSSLQDKSEYLEMEEGLEKESLC
 CTGAAAAAGTTGAGGAGCTCCACATCTCTCTCTCAGGACAAATCTGAATATCTAGAGATGAGGAGAGGGCTCAAAGAGTCTCTCTGT

 2701 VKDKAIEGTGNGNETIKYNCVSLKILELTDV
 GTAAAGACAAAGCTATTGAGGGACAGGGAATGGCAAGGACCAATTAAGTATAACTGTGTGAGTGTAAAAATCTGGAACCTGATGTG
 2791 TGAATATATATTTTGGACCTTCTCAGAGCTTATTAATGATCAAGAGTGGAGTATGAGCAAGAAATGAATATGCAATCTAATCAAGA
 2881 AATGGCAGCTGCTTCAATGATGTGTAATACAGGCGCTCTCTGTTGATGAGATACACAAATGGGACAGAGTCAAGTCTGAA
 2971 GATAGCAGTGGGCTTCTGCTGGCTTTTCTCTAAAGTGAAGTGTGTCACCATAGATATAATATGCAAGTGTGACATCTGAC
 3061 TATTTCTACTTTAGAGAGGGGTACATAGTGTATTAATATGAGGAGAGAAACCATGGAAGAGACAGCAGTATTTTTTGTACATG
 3151 ACTGCTATGTTTCAAATAATACCTGGCACCGGATAAATACATTTGTTTACTTGTGTTGCTATGAACATTTATTTTTTATTAAGATGTT
 3241 TATTTCTCAAAGATTCACATCAAAGATAA

BALL

XKv1.4 : MEVAMVSADSSGCSNHLPGYA--QARARERERARSRATAAAASGEGGNSCGGAGVNARRAPQOVFERQEEKSSQKKKSA 81
 mKv1.4 : MEVAMVSADSSGCSNHLPGYA--QARARERERARSRATAAAASGEGGNSCGGAGVNARRAPQOVFERQEEKSSQKKKSA 79
 cKv1.4 : MEVAMVSADSSGCSNHLPGYA--QARARERERARSRATAAAASGEGGNSCGGAGVNARRAPQOVFERQEEKSSQKKKSA 82
 ShakerB: MAAVAGLYGLGEDRQRRKQQQQQQ-----HQRQ-----LEQKEEQKKIAE 42

CaMKII

XKv1.4 : RRRYWPLSGCNWBSRHNECSGGAGGRAGGEDDCTFT--SEGLGCSSEWMLR-----PVAEEDDKPFICEE 148
 mKv1.4 : RGRRRRR--RQTEKKKLH-----HQQS-----SPFHCSDLMP--SSEERKILRELSEEEE--DEEEEEEEEGGPHYSE 145
 cKv1.4 : RSSLNRSQSGKMMKGGKGRSH-----HLGREGCAGSPF--CSELLPLSGSEERHIAOVSEEEEEDEDEDDDEEGKILYSAD 158
 ShakerB: R-----RLQLRQQQLQ-----RNSLDGYCSL-----RLSSQDE 171

Tyr K T1

XKv1.4 : DEKEANLHRRRSPTEDF--VHPY-YSEFECERVVINVSGMYETQTKTLFOPPETLLGDPFKRTRYFDFPLRNEYFFDRN 226
 mKv1.4 : DHGDCGCTDLLPQEDGGGGGSSVRYS--CCERVINVSGLRFTQTKTLAOPPETLLGDEPKRTQYDFPLRNEYFFDRN 225
 cKv1.4 : YGEDEFSGYDGPDDGGGGGSSVRYSYETECERVVINVSGLRFTQTKTLAOPPETLLGDPFKRGRYDFPLRNEYFFDRN 240
 ShakerB: EGGAGHCGG-----GGPQHEEPHDPH--DFCERVINVSGLRFTQTLRTLAOPPETLLGDEPKRTRYFDFPLRNEYFFDRS 145

S1 N-Gly S2

XKv1.4 : RGLAIVSVLVILISIVIFCLETLPFRDDKDNLSLPGHGGDDGAGEDGEGGAYNATFLSTDSGHTAFNDPFFIVETVCIVW 389
 mKv1.4 : RGLAIVSVLVILISIVIFCLETLPFRDDK--LIMALSAGGHSRL-----NDTSAPHLDSGHTAFNDPFFIVETVCIVW 382
 cKv1.4 : RGLAIVSVLVILISIVIFCLETLPFRDDKE--FIMSLSSC--KGIS-----NESLHDDGCHTIFNDPFFIVETVCIIW 393
 ShakerB: RVVAISVVFVILLSIVIFCLETLPFRHRYGV--FNTTNG-----TKREDEVPDITDPFFLIETLCIIW 289

S3

XKv1.4 : FSFEAVRLFACPSNPPSKNIMNIIDIVSILPYFITLGTREPQQHPQQQQHLALVTGQQLPGTGQQQQQAMSFAILRIIR 471
 mKv1.4 : FSFEAVRRCFACPSNPPSKNIMNIIDIVSILPYFITLGTADLQQQQGG-----NGQQQAMSFAILRIIR 448
 cKv1.4 : FSFEAVRRCFACPSNPPSKNIMNIIDIVSILPYFITLGTADLQEQGS-----NGQQ--AMSFAILRIIR 456
 ShakerB: FTTELTVRFLACPSNPPSKNIMNIIDIVSILPYFITLGTAVVAEEDTLN-----LPKAPVSPQKSSNQAMSFAILRIIR 365

S4 PKC S5 PORE

XKv1.4 : LVRVFRIFKLSRHSKGLQILGHTLRASMRELGLLILFLFIGVILFSSAVYFAEADFPATHFQSIDPAFWAVVMTTIVGYGD 553
 mKv1.4 : LVRVFRIFKLSRHSKGLQILGHTLRASMRELGLLILFLFIGVILFSSAVYFAEADFPATHFQSIDPAFWAVVMTTIVGYGD 530
 cKv1.4 : LVRVFRIFKLSRHSKGLQILGHTLRASMRELGLLILFLFIGVILFSSAVYFAEADFPATHFQSIDPAFWAVVMTTIVGYGD 538
 ShakerB: LVRVFRIFKLSRHSKGLQILGHTLRASMRELGLLILFLFIGVILFSSAVYFAEAGSENSFPKSIDPAFWAVVMTTIVGYGD 447

S6 Tyr K PKA

XKv1.4 : MKPITVGGKIVGSLCAIAGVLTIALPVPVIVSNFNFYFHRETDNEE--QTOLTSSSSCPYLPNTLLKKLR---SSTSSSLQ 630
 mKv1.4 : MKPITVGGKIVGSLCAIAGVLTIALPVPVIVSNFNFYFHRETDNEE--QTOLTANAVSCPYLPNTLLKKLR---SSTSSSLG 607
 cKv1.4 : MKPITVGGKIVGSLCAIAGVLTIALPVPVIVSNFNFYFHRETDNEE--QTOLTANAVSCPYLPNTLLKKLR---SSSSSTE 615
 ShakerB: MKPITVGGKIVGSLVAGVLTIALPVPVIVSNFNFYFHRETDREDAQSNFNHVTSSVILGALQHLKSSLSSESSSLM 529

Tyr K

XKv1.4 : DKSE-----YLEMEEGKLESQVNDKALIGTGNG-----NETHKNVNSLKI 672
 mKv1.4 : DKSE-----YLEMEEGVKSLECGHEKCCGCKGD-----SETDKNCSNAKA 649
 cKv1.4 : DKSE-----YLEMEEGVKSLECGHEKKSQDENS-----SESPKNVNSNS 657
 ShakerB: DLDDGIDATTPGLTDHTGRHMVFRTGCSFEKQQLQLQLQLQQSSPHGQQTQQQLGQNGLRSTNSQLRHNAMAVS 611

XKv1.4 : LETDV 677
 mKv1.4 : VETDV 654
 cKv1.4 : VETDV 662
 ShakerB: LETDV 616

which flanked the predicted coding region yielded a 2.2 kb product which confirmed the sequence of the overlapping clones. Analysis of the 5' untranslated region (UTR) cDNA sequence showed that it possessed five potential MyoD binding sites. Translation of the cDNA sequenced revealed an open reading frame of 2031bp that potentially encoded a 677 amino acid protein similar to Kv1.4 from other species (Figure 3.2B). The cDNA was designated XKv1.4. The putative amino acid sequence of XKv1.4 was 73% similar to mouse Kv1.4; 74% similar to chick Kv1.4, and 74% similar to the *Shaker B* K⁺ channel. The predicted extracellular S1-S2 linker of the XKv1.4 protein was 12 amino acids longer than those of mouse and chick Kv1.4 channels. In addition, the S3-S4 linker of XKv1.4 was 18 amino acids longer than those of mouse and chick Kv1.4. The *Shaker B* K⁺ channel also possessed an S3-S4 linker which was 11 amino acids longer than mouse, but it had little similarity to the extra 18 amino acids of XKv1.4. The S1-S2 potential N-linked glycosylation site, the S4-S5 potential PKC site, the C-terminal potential PKA site and the N-terminal CaMKII site are all well conserved in the Kv1.4 isoforms, and were found to be present in the XKv1.4 protein (Figure 3.2B). XKv1.4 also had three potential sites for modification by tyrosine kinase phosphorylation. In addition, the C-terminal T/SXV motif, thought to be important in regulating membrane expression (Kornau *et al.*, 1995; Jugloff *et al.*, 2000) was also present in XKv1.4 (Figure 3.2B).

The XKv1.4 predicted protein sequence isolated in the present study contained the hallmark "ball and chain" domain of the vertebrate Kv1.4 inactivating K⁺ channels (Armstrong and Bezanilla, 1977; Hoshi *et al.*, 1990; Zagotta *et al.*, 1990; Jan and Jan, 1992). Within the predicted ball domain (amino acids 1-36) XKv1.4 showed approximately 90% identity with mouse and chick Kv1.4 channels, but all three showed significant divergence from each other in the chain domain (Figure 3.2B). Furthermore, the mouse and chick Kv1.4 chain domains are characterized by a number of repeated glutamic acid residues which are absent from XKv1.4. These data suggest the presence of Kv1.4 K⁺ channel mRNA in *Xenopus* muscle which may encode a rapidly inactivating K⁺ channel.

XKv1.10 encodes a novel Kv1 channel

Three overlapping clones obtained by RT-PCR, 3' and 5' RACE ultimately produced 2033 bp of cDNA (Figure 3.3). RT-PCR with primers 10-D and 10-E which flanked the predicted coding region yielded a 1519 bp product which confirmed the sequence of the overlapping clones. Analysis of the 5' untranslated region (UTR) cDNA sequence showed that it possessed one potential MyoD binding site. Translation of the cDNA sequenced revealed an open reading frame of 1467 bp that potentially encoded a 489 amino acid protein (Figure 3.3). The predicted protein possessed domains characteristic of Kv channels including six putative membrane spanning domains, a subunit

Figure 3.3. Analysis of the XKv1.10 cDNA. Nucleotide sequence of the cDNA for XKv1.10, and the deduced amino acid sequence. Potential MyoD binding sites (CANNTG) in the 5' UTR are shaded in gray. The position of the tetramerization domain (T1), membrane spanning domains (S1-S6) and pore domain are indicated. Potential phosphorylation sites by protein kinase C (PKC) and protein kinase A (PKA) are indicated. Potential MyoD binding sites (CANNTG) in the 5' UTR are shaded in gray.

1 AGGCTTAATCGAGTTAGGAAGACCTGAAATCTTCATCTCTGTGGATCTGTTACGACACAGCGAGCTACTGCGAGCCATATGAGAAA
 91 GAAGGCAAGAAAGGTTGTGATCTGAGAAAGGAAGTGAGTAAAGCATTATGTTTCATTCGAGAAAGAGGTAATAGATTGATTCAA
 181 AGGAAGGAAGGCAAGGTAACGTAAAGGGGACTGAGAAAGACACAGTTACCATGATACATAAATGATTATTCCCATGTAAAGCTG
 271 AAGCCAAAGCTCATGAAGAATTCATCAATCCAGTTCAGGCTATGAGTATGTTAAACCATTCAGCAACAACCTAGGAATACC

Tyr K
*

M P S V M E N G D E V T G H S N L A L I P D Q K K Y E I C 29
 361 AATATGCCCTCGGTATGAGAGAGGGGATGAAGTACTGGAACCTCAACCTTGCCGTATCCAGACAGAGAAGATGAAATCTGC

R Q K N C C E R V V I N V S G L R F E T T Q V R T L S R F P D 59
 451 CGACAAAGAAATGCTGTGAGCGGGTGGTGATCAATGTGTCTGGACTACGGTTTGAGACCCAAAGTCAGAGCCCTCAGTCGGTTCTCGTAT

T1

T L L G D P R R R I H F F D P L R N E Y F F D R N R P C F D 89
 541 ACTCTGCTGGGAGCCCAAGGAGGAATACACTCTTTGATCCCTTGAGGAATGAGTACTCTTTGATCGAAACCGACCATGCTTTGAC

S I L F F Y Q S G G R L R R P P N V F L D V F M E E L L F Y 119
 631 TCAATACCTTTCTTTCATGCTCGGGGGGTGCTCTCGCGGGCCACCAATGTCCTTTAGATGCTTTATGGAGGAGCATTAATTTAT

E L G Q D V M M K F R E D E G F M K E E E R L L P E C E F M 149
 721 GAGCTCGGACAGGATGTAGTCATGAAATTCGGGAGAGATGAGGGATTTATGAAGAGGAGGAAGACTCTCCAGCAATGTGAGTTCATG

S1

K Q V W L L F E H P D S S S A A R I I A I I S V M H V I L I S 179
 811 AAGCAGGTCTGCTTCATTTGAAGTACAGATAGCTCATCTGCGCAAGATCATTCGCAATATCTCTGTATGTCATCTTCATCTCC

I V I F C L E T L P E F R D D R D V S L P V H L H R A N S T 209
 901 ATTGTCTCTCTGCTTGAACACACTACCAGAAATCAGAGATGACAGGATGTATCTTACC GGTCATCTTCATCGGCTAACGACACA

S2

H H L K E K S P F E D P F F F I V E T I C I C W F S F E L S V 239
 991 CACCATTGAGGAGAAAAGCCCTTCGAGGACCCCTTTTATTTGAGGAGCATCTGTATCTGCTGGTTTTCCTTTGAGCTTTCAGTG

S3

R F F A C P S K A A F F K D I M N M I D F V A I I P Y F V A 269
 1081 AGGTTTTTTCATGCCCTAGCAAGGCGAGCTTTCTCAAGGACATAATGAACATGATGATTTTGTGGCCATCATACCTATTTGTTGGG

S4

L G T E L A R N K G V G Q P A M S L A I L R V I E L V R V F 299
 1171 CTGGGTACTGAAGCTGCTGCAACAAAGGTGAGGGCAACCTGCTATGCTTTGGCCATCTCAGGGTATTCGGTTTGGTCCGAGCTTC

PEC
*

R I F K L S R H S K G L Q I L G Q T L K A S M R E L G L L I 329
 1261 CGTATCTTTAACTCTCCCGTCACTCTAAGGGTTTACAGATCTTGGGACAGACATTGAAAGCCAGTATGAGGGAATTTGGGCTCTTGATA

S5

F F L F I G V I L F S S S V Y F A E A D H A D T A F T S I P 359
 1351 TTTTCTCTTTTCATCGGAGTTATCTCTCTCGAGCTCAGTCTACTTTGCTGAGGCTGATCATCGGATACAGCGTTTACAAATGATCCCT

PORE

E A F W W A V V T M T T V G Y G D M S P E T V G G K L V G S 389
 1441 GAAGCATTTTGGTGGGCTGTGGTCAACATGACCAAGTATGAGATATGTCACCTGAAACAGTAGGTGGGAACTGGTGGGCTCT

S6

L C A I A G V L T I S L P V P V I V E N F S Y F Y H R E T E 419
 1531 CTTTGTGCCATTGCGGGTGCTGACCATCTCTGCGAGTGCCAGTCAATGTATGCAATTTAGTTATTTTCTACCACGAGAACTGAA

PKA
*

C S D I G Q Y D H V G S C F N S P P L T P K T K G R D K S P 449
 1621 TGTCTGATATTGGCCAGTACGACCATGTTGGGTCTGCGCTAATAGTCTCCACTTACCCCAAGAAAGCAAGGAGGAGGAGTGTCT

M I S K L R N I V T N S K A E F L D G L G P A D D R Q L V P 479
 1711 ATGATAAGCAACTTGAATATAGTCAACCAATCCAGGCGAAGTCTCGATGGACTAGGCGCTGCTGATGACAGACAGCTGGTCTCT

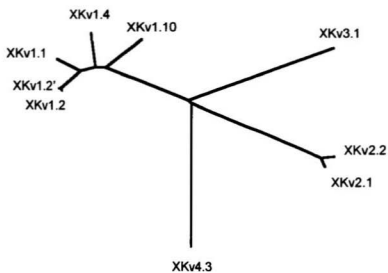
G Q P S H L I T E V 489

1801 GGCAGGCTCATCATTTGATAACCGAAGTTGATTTTGGAAATGCTTCACTTAATCTGCTCTTCAACCTTGTCTTAAATATAACAT
 1891 TACATGAAGAGTCAAGCAAGATGATTTCTTTTATCTTGGATTTCACCAAGAAACCTGTTTCATTGAGACACCTCATCCAGAGAC
 1981 GTTGCAATTTTCTCTGGGCTTTAAATAATGTTGATTANTATAAAGTTGTC

tetramerization domain, and a pore domain containing the signature sequence of SMTTVGYG (Heginbotham *et al.*, 1994). Overall, the novel protein shared 79% similarity with XKv1.2' and 72% with XKv1.4, but shared about 40% similarity with either XKv2, XKv3 or XKv4 proteins. Its putative tetramerization domain shared 89% similarity with XKv1.2', and 86% with XKv1.4. The hydrophobic core region (S1-S6) of the predicted protein shared 79% similarity with XKv1.2 and 72% with XKv1.4. The pore region of the predicted protein shared 95% with XKv1.2 and 94% with XKv1.4. The intracellular N and C-terminal regions and the extracellular S1-S2 and S3-S4 loops of the novel protein however showed considerable divergence from other known members of the Kv1 family. Phylogenetic analysis of the predicted protein, combined with these data suggested that we have isolated a cDNA which encodes a novel Kv1 channel (Figure 3.4), and we have designated the K⁺ channel protein encoded by this cDNA XKv1.10.

Although XKv1.10 also had potential sites for post-translational modification (Figure 3.3), the site for N-linked glycosylation in Kv1 channels which is usually found in the S1-S2 linker was absent from XKv1.10. There were potential sites for phosphorylation, including a potential site for PKC in the S4-S5 linker, a potential site for PKA in the C-terminal about 40 amino acids from the S6 domain and a potential site for tyrosine phosphorylation near the N-terminal. In contrast to the XKv1.2' and XKv1.4 predicted proteins, there were

Figure 3.4. Relationship of XKv1.10 to other *Xenopus* Kv channels. The predicted amino acid sequences between the T1 and S6 domains from the known *Xenopus* K⁺ channels were aligned using Clustalw, and an unrooted phenogram was constructed using the MEGA2 software package. The distance between two K⁺ channels represents extent of divergence.



no potential sites for tyrosine kinase phosphorylation in the C-terminal region of XKv1.10. The TEV terminal amino acid sequence of XKv1.10 conforms to the expected T/SXV for a Kv1 subfamily member. These data suggest the presence of a previously unknown channel, XKv1.10, in *Xenopus* muscle.

3.3.2 Functional expression of XKv1.2', XKv1.4 and XKv1.10

Preliminary characterization of the XKv1.2', XKv1.4 and XKv1.10 channels was carried out in HEK 293 cells that express only very low density endogenous voltage gated K⁺ currents (Uebele *et al.*, 1996; Figure 3.5). All three cDNAs encode voltage gated K⁺ channels with differing characteristics.

XKv1.2' encodes a non-inactivating voltage activated K⁺ channel

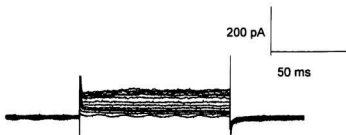
Figure 3.6A shows a family of currents elicited by a series of 100 ms depolarizing pulses recorded from a HEK 293 cell co-transfected with GFP and XKv1.2'. The current activated rapidly, and did not show significant inactivation over the 100 ms pulse. The voltage of activation was between -40 mV and -30 mV (Figure 3.6B). As this was preliminary data, voltage of half activation for this channel and the others was not determined. XKv1.2' channels were insensitive to 40 mM TEA, however current was reduced by 30% -100% by 300 μ M 4-AP (n= 5, Figure 3.6C).

XKv1.4 encodes a rapidly inactivating channel

Figure 3.7A shows a family of currents elicited by a series of 100 ms depolarizing pulses recorded from a HEK 293 cell co-transfected with GFP and

Figure 3.5. Currents elicited from HEK293 cell transfected with only GFP construct. A. The cell membrane potential was held at -80 mV, and depolarized from -70 mV to +50 mV in 10 mV steps. B. Mean current density-voltage relationship showing that HEK 293 cells have very little endogenous K^+ current. Current was not leak subtracted.

A



B

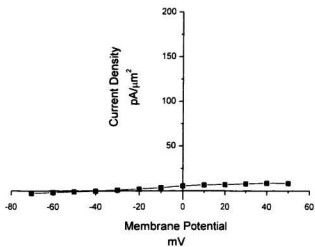
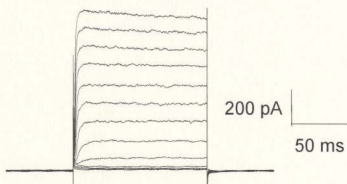
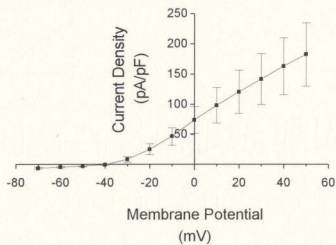


Figure 3.6. Current traces from HEK 293 cell co-transfected with GFP and XKv1.2'-pCDNA3 constructs. A. The cell membrane potential was held at -80 mV, and depolarized from -70 mV to +50 mV in 10 mV steps. The current does not demonstrate inactivation. B. Mean current density-voltage relationship of HEK 293 cells expressing the XKv1.2' construct (n = 8; error bars SEM). Threshold for current activation is between -40 mV and -30 mV. C. XKv1.2' is blocked by externally applied 30 μ M 4-AP. Superimposed current traces from a voltage clamped HEK 293 cell transfected with pCDNA3-XKv1.2' depolarized to 30 mV in the absence (control) and presence of 300 μ M 4-AP. Currents were not leak subtracted.

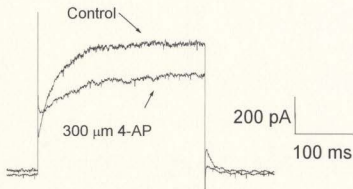
A



B



C



Kv1.4. The current activated rapidly, reaching peak current in < 6 ms at +50 mV, and showed significant inactivation (49%) over the 100 ms pulse at this potential ($\tau = 52.1 \pm 6.0$ ms, $n = 4$). The voltage of activation was between -40 mV and -30 mV (Figure 3.7B). Sensitivity of XKv1.4 channels to TEA and 4-AP was not tested.

XKv1.10 encodes a rapidly inactivating channel

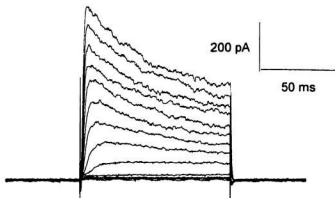
Figure 3.8A shows a family of currents elicited by a series of 100 ms depolarizing pulses recorded from a HEK 293 cell co-transfected with GFP and XKv1.10. The current produced by XKv1.10 was almost identical to that of XKv1.4 channel, showing rapid activation, reaching peak current in < 6ms and inactivation (22.5%) over the 100 ms pulse ($\tau = 46.3 \pm 4.9$ ms at +50mV, $n = 3$). The voltage of activation occurred between -30 mV and -20 mV (Figure 3.8B). Sensitivity to TEA and 4-AP was not tested.

3.3.3 XKv1.2', XKv1.4 and XKv1.10 show unique patterns of expression during development

The developmental expression of XKv1.2', XKv1.4 and XKv1.10 RNA was examined in whole embryos and tadpoles (Figure 3.9 A). XKv1.2' transcripts were first detected during late gastrulation (stage 11.5) where the intensity of the signal was weak. The intensity of the RT-PCR product for the XKv1.2' transcript however increased through development, and was detectable through to stage 50. XKv1.4 transcripts were first detectable during early gastrulation (stage

Figure 3.7. Current traces from HEK 293 cell co-transfected with GFP and XKv1.4-pCDNA3 constructs. A. The cell membrane potential was held at -80 mV, and depolarized from -70 mV to +50 mV in 10 mV steps. The current demonstrates inactivation (τ value for current at 50 mV = 43.4 ms). B. Peak (squares) and late (circles) current density-voltage relationship of HEK 293 cells expressing the XKv1.4 construct ($n = 3$; error bars SEM). Threshold for current activation is between -40 mV and -30 mV. Current was not leak subtracted.

A



B

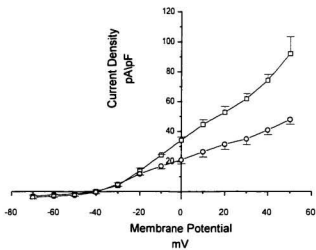
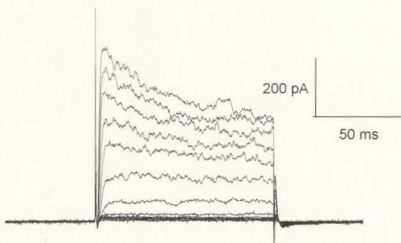


Figure 3.8. Current traces from HEK 293 cell co-transfected with GFP and XKv1.10-pCDNA3 constructs. A. The cell membrane potential was held at -80 mV, and depolarized from -70 mV to +50 mV in 10 mV steps. The current demonstrates inactivation (τ value for current at 50 mV = 46 ms). B. Peak (squares) and late (circles) current density-voltage relationship of HEK 293 cells expressing the XKv1.10 construct ($n = 3$; error bars SEM). Threshold for current activation is between -30 mV and -20 mV. Current was not leak subtracted.

A



B

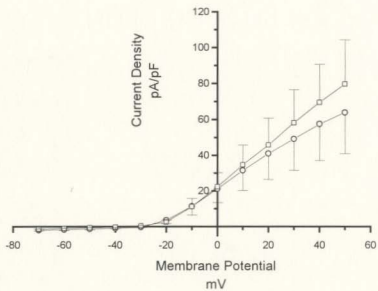
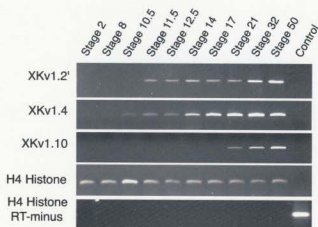


Figure 3.9. A. Developmental expression of XKv1.2', XKv1.4 and XKv1.10 in whole embryos. cDNA was prepared from whole embryos at stages 2, 8, 10.5, 11.5, 12.5, 14, 17, 21, 32 and 50 and XKv1.2', XKv1.4 and XKv1.10 expression was assessed using specific primers as described in the Methods.

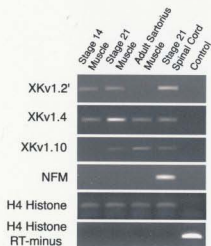
B. Expression of XKv1.2', XKv1.4 and XKv1.10 in muscle and nerve. cDNA was prepared from the muscle of stage 14 and 21 embryos, adult sartorius muscle and stage 21 spinal cord. Expression of XKv1.2', XKv1.4 and XKv1.10 RNA was assessed using specific primers as above. Additional RT-PCR with primers specific for medium weight neurofilament (NFM) was carried out to demonstrate that the muscle RNA sample was uncontaminated by nerve.

Controls RT-PCR with primers for H4 histone was carried out with each of the cDNA preparations as a positive control. The control lane at the right of the figure for the Kv1.2', Kv1.4 Kv1.10 and H4 reaction contained no template. The H4 primers were used with RT-minus template to ensure there was no genomic DNA and the control lane for this series contained stage 50 cDNA.

A



B



10.5), prior to detection of the XKv1.2' transcripts, and continued to be expressed through stage 50. Transcription of XKv1.10 was delayed compared to that of XKv1.2' and XKv1.4, as it was not detectable until stage 21. The intensity of the XKv1.10 RT-PCR product also increased as the embryo matured to tadpole (Figure 3.9A).

The specific expression of each of the three sequences was examined in embryonic and adult muscle, and embryonic spinal cords (Figure 3.9B). XKv1.2' transcripts were detected at low levels in stage 14 muscle, were still present in stage 21 muscle but were not detected in adult sartorius muscle. XKv1.4 transcripts were also detected in stage 14 muscle, were more apparent in stage 21 muscle and were also expressed in adult muscle. XKv1.10 transcripts were not detected in stage 14 muscle but were detected in both stage 21 muscle and adult sartorius muscle. All three K⁺ channel transcripts were also expressed in stage 21 spinal cord. XKv1.2' had a higher expression level in spinal cord than in muscle whereas both XKv1.4 and XKv1.10 transcripts were more highly expressed in muscle rather than spinal cord. The medium weight neurofilament transcripts, specific for neurons (Gervasi and Szaro, 1997) was present in the spinal cord preparation, but absent from the muscle preparations demonstrating the absence of neuronal contamination.

3.4 Discussion

The aim of the present study was to determine the molecular identity of voltage gated K^+ channels expressed in *Xenopus* myocytes during development which may underlie the K^+ currents observed by electrophysiology. Here we report the cloning of three cDNAs from *Xenopus* muscle which encode K^+ channel proteins. One of these cDNAs, XKv1.2', encodes a channel protein that is 98% identical to a previously reported *Xenopus* K^+ channel, and produces a non-inactivating voltage activated K^+ current with nearly identical biophysical properties (Ribera, 1990). The second cDNA, XKv1.4, encodes a Kv1.4 channel protein isoform, complete with the distinctive "ball and chain domain" (Hoshi *et al.*, 1990), and this channel produces inactivating currents (Zagotta *et al.*, 1990). The third cDNA, XKv1.10, encodes a novel Kv1 isoform, which produces an inactivating current similar to that of XKv1.4. The results of the present study also demonstrate that these K^+ channel genes are differentially expressed during development of muscle, and that they are also expressed in early spinal cord neurons.

3.4.1 The cDNA sequences encode K^+ channels.

Results of the sequence analysis show that the three cDNAs isolated from muscle encode K^+ channels. All three cDNAs predict proteins containing amino acid sequences corresponding to the well-conserved hydrophobic membrane

spanning domains S1 to S6, the subunit tetramerization domain T1, and the characteristic K⁺ channel pore domain, P1 (Heginbotham *et al.*, 1994). XKv1.2' and XKv1.4 were identified as K⁺ channels based on the level of homology with other respective Kv1.2 and Kv1.4 cDNAs. The third K⁺ channel cDNA, XKv1.10, was more closely related to the Kv1 subfamily than any other group of K⁺ channels, but did not appear to possess similarity to any Kv1 isoforms previously described from other species (see below). Both Kv1.2 and Kv1.4 isoforms have been shown to produce functional K⁺ channels when expressed in oocytes (Stuhmer *et al.*, 1989; Ribera, 1990), however the ability of XKv1.10 to encode a functional K⁺ channel is yet to be demonstrated. The predicted XKv1.10 protein however does contain the domains required for Kv channel function.

XKv1.2'

The protein encoded by the XKv1.2' cDNA isolated in the present study by RT-PCR is virtually identical to the XKv1.2 clone isolated by Ribera (1990). Given that most vertebrate Kv1 genes are found at only a single locus (Chandy and Gutman, 1995) we suggest that the XKv1.2' cDNA isolated here is an allelic variation of XKv1.2, not a new gene. Southern blot analysis with an XKv1.2 probe revealed a hybridization pattern which was consistent with a single XKv1.2 gene (Ribera, 1990). Furthermore, the variation between the XKv1.2 and XKv1.2' sequences is consistent with the pseudotetraploidy of the *Xenopus* genome (Kobel and Du Pasquier, 1986) where a given gene can have up to four

alleles. Similar minor variations in other *Xenopus* Kv channels have previously been noted (Burger and Ribera, 1996; Gurantz *et al.*, 1996)

Based on the similarity of XKv1.2' to XKv1.2, it was expected that XKv1.2' would also encode a channel that produces a non-inactivating K⁺ current (Ribera, 1990). Indeed, the current density-voltage relation (Figure 3.6B) showed that the current produced by XKv1.2' had a threshold of activation between -40 mV and -30 mV. This is similar to that recently reported for XKv1.2 (Ribera, 1990; Ribera and Nguyen, 1993). Moreover, the very slow inactivation kinetics (Figure 3.6A) is similar to that of rat Kv1.2 (Stuhmer *et al.*, 1989) and XKv1.2 (Ribera, 1990). The insensitivity of XKv1.2' to TEA, and sensitivity to 4-AP is also similar to that of rat Kv1.2 (Stuhmer *et al.*, 1989) and XKv1.2 (Ribera, 1990).

XKv1.2' is expressed in *Xenopus* myocytes grown in culture, and thus the current produced by XKv1.2' may underlie the observed non-inactivating I_K current observed in this preparation (Moody-Corbett and Gilbert, 1992b; Spruce and Moody, 1992; Ribera and Spitzer, 1990).

XKv1.4

The second K⁺ channel cDNA encodes a Kv1.4 homologue. A feature unique to the Kv1.4 isoform is the presence of a "ball and chain" domain which confers its rapidly inactivating kinetics (Armstrong and Bezanilla, 1977; Zagotta *et al.*, 1990; Hoshi *et al.*, 1990). The 35 N-terminal amino acids of the deduced

XKv1.4 peptide appear to be a ball domain as it shares significant homology with the ball domains of other Kv1.4 homologues (Stuhmer *et al.*, 1989). Whereas the ball domains of mouse, chick and *Xenopus* Kv1.4 homologous are highly conserved, the chain regions show considerable divergence. This lack of conservation in the chain domain may not have any effect on the ability of the XKv1.4 to produce an inactivating current, as deletion of the amino acids 28-283 of a mammalian Kv1.4 did not significantly alter the rate of inactivation (Lee *et al.*, 1996). Significant divergence between mKv1.4, cKv1.4 and XKv1.4 was also noted in the S1-S2, and S3-S4 extracellular loops, where XKv1.4 shows a number of inserted amino acids; however the significance of these inserts has yet to be determined.

Based on the presence of the inactivating ball domain, and the overall similarity to other Kv1.4 channels, it was predicted that XKv1.4 would produce a rapidly inactivating current. The electrophysiological data showed that XKv1.4 encodes a channel with inactivating kinetics similar to other Kv1.4 channels. The inactivation time constant of $\tau = 52.1 \pm 6.0$ ms is similar to the 50 ± 25 ms reported by Kupper, (1998) and $69 \text{ ms} \pm 23$ reported by Petersen and Nerbonne, (1999) recording rat Kv1.4 in HEK 293 cells, although Petersen and Nerbonne also reported a second component of inactivation with $\tau = 324 \pm 116$ ms. Other reported τ values for the inactivation of Kv1.4 in HEK 293 cells are slightly longer: 132 ± 34 ms (Jugloff *et al.*, 2000).

The activation potential (between -40 and -30 mV) of XKv1.4 was also somewhat depolarized to that of rat Kv1.4 in some reports (-55 mV reported by Stuhmer *et al.*, 1989), but appears to be similar to that of rat Kv1.4 in other reports (see Figure 5, Jugloff *et al.*, 2000). The differences in activation parameters may lie in expression system (Peterson and Nerbonne, 1999). Based on the preliminary data, XKv1.4 appears to behave similarly to other Kv1.4 channels in spite of the significant differences in the chain domain and the extracellular domains. Of course, more detailed analysis of more transfected HEK cells will be required to confirm this. Additionally, caution should be used in comparing channels from different expression systems, as different heterologous systems are known to affect their biophysical parameters (Petersen and Nerbonne, 1999).

This is the first report describing the isolation of a Kv1.4 orthologue from *Xenopus*, and the following lines of evidence suggest that the channel encoded by XKv1.4 contributes to the inactivating current, I_{K1} , reported in *Xenopus* myocytes in culture (Chapter 2; Moody-Corbett and Gilbert, 1992a,b; Spruce and Moody, 1995; Linsdell and Moody, 1995). Firstly, the Kv1.4 isoform is well known to produce an inactivating current (Stuhmer *et al.*, 1989; this study). Secondly, the voltage of activation for XKv1.4 (-40 to -30 mV) was similar to that reported for I_{K1} in *Xenopus* myocytes in culture (Chapter 2; Moody-Corbett and Gilbert, 1992a,b). Thirdly, the present study demonstrates the presence of

XKv1.4 mRNA in *Xenopus* myocytes at times when the inactivating current is known to be present. I_{IK} is reported in myocytes isolated from stage 14-15 embryos (Spruce and Moody, 1992) and stage 19-21 embryos (Chapter 2; Moody-Corbett and Gilbert, 1992a,b) as well as in acutely dissociated tadpole tail muscle cells (unpublished observations). Finally, Ernsberger and Spitzer (1995) have found that channels that mediate I_{IK} in *Xenopus* myocytes are sensitive to reducing agents. Channels encoded by Kv1.4 are known to be sensitive to reducing agents by a mechanism involving a cysteine residue in the ball domain (Ruppersberg *et al.*, 1991). Consistent with this, XKv1.4 described in the present study contains a cysteine residue at position 14. Taken together, these studies and the present findings provide evidence that the inactivating current seen in *Xenopus* myocytes is mediated in part by XKv1.4. This interpretation does not discount the possibility that channel inactivation is also mediated by a non-inactivating α -subunit associated with inactivating β -subunits. However, it is worth noting that none of the β -subunits isolated in a thorough *Xenopus* library screening, which are present in skeletal muscle, impart inactivating characteristics to K^+ currents when co-expressed with α -subunits in oocytes (Lazaroff *et al.*, 1999). Therefore, we propose that the XKv1.4 channel isolated here contributes to I_{IK} observed in *Xenopus* myocytes.

XKv1.10

This is the first report of the K^+ channel gene we have designated

XKv1.10 using the nomenclature of Chandy and Gutman (Chandy and Gutman, 1993). XKv1.10 bears the most significant similarity (about 70%) with members of the Kv1 subfamily, and about 40% with Kv2, Kv3, and Kv4 subfamilies. As expected, most of the similarity occurs within the hydrophobic core.

The currents recorded from HEK 293 cells transfected with Kv1.10 constructs showed rapid inactivation with kinetics similar to those of the XKv1.4 channel. This was unexpected as the predicted protein did not contain a domain analagous to an inactivation ball. Therefore, the rapid inactivation must be mediated by a rapid C-type inactivation process as has been previously described for Kv1.7 (Kalman *et al.*, 1998).

The observation that the potential of threshold of activation for the XKv1.10 channel was somewhat depolarized (occurring between -30 and -20 mV) together with the observation that XKv1.10 expression was not detected until some time between stage 17 and stage 21 (see below), strongly suggests that XKv1.10 does not contribute to an early appearance of I_{K1} (Spruce and Moody, 1992). However it is likely to contribute to I_{K1} at later ages. Given the level of expression in adult skeletal muscle, it is possible that this cDNA encodes a channel that produces a significant portion of the voltage gated K^+ current in mature *Xenopus* skeletal muscle, and is partially responsible for the observed inactivating component of the K^+ current (Adrian *et al.*, 1970a).

3.4.2 The expression of XKv1.2', XKv1.4 and XKv1.10 are developmentally regulated

Developmental regulation of K⁺ channel subtypes is complex and specific.

As discussed below, all three of the K⁺ channel genes isolated here have unique patterns of expression. Therefore, it is likely that they contribute to the maturation of electrical excitability in *Xenopus*.

XKv1.2' expression initiated at stage 11.5, and it was still expressed at stage 50. XKv1.2' was detectable in embryonic muscle at the earliest time muscle was identifiable and also in muscle tissue at stage 21. However XKv1.2' was not expressed in mature frog muscle. Relative to muscle, XKv1.2' is quite strongly expressed in stage 21 spinal cord. Similarly, Ribera (1990) reported that XKv1.2 was detectable at stage 13 in the dorsal posterior third of embryos, and became strongly expressed in neural tissue thereafter. In contrast, Ribera (Ribera, 1990) could not detect XKv1.2 in skeletal muscle. The most likely explanation for this is the high sensitivity of PCR compared to the RNase protection assay used by Ribera. The expression pattern of XKv1.2' observed here is consistent with that reported for embryonic rat where it is found that Kv1.2 is expressed at low levels in embryonic day 16-18 and early post natal rat skeletal muscle, but is not detectable in adult rat skeletal muscle (Lesage *et al.*, 1992). In addition, expression of Kv1.2 was quite strong in both *Xenopus* and rat nervous tissue during early development (Lesage *et al.*, 1992).

This is the first report of the developmental expression of a Kv1.4 orthologue in *Xenopus*. XKv1.4 expression initiated at early-gastrulation, and it was still expressed at the tadpole stage. As described for both human (Philipson *et al.*, 1990) and rat (Lesage *et al.*, 1992) skeletal muscle, Kv1.4 was expressed specifically during muscle cell development. In the present study XKv1.4 was expressed in embryonic and mature muscle but was most strongly expressed in stage 21 muscle. These data are also consistent with Lesage *et al.*, (1992), who reported that Kv1.4 is moderately expressed in developing skeletal muscle, but expressed at lower levels in mature muscle. In addition XKv1.4 was expressed at low levels in spinal cord neurons at stage 21.

The novel XKv1.10 is expressed in whole embryos starting at stage 21, and is still expressed at the tadpole stage. The transcript was weakly expressed in myotomal muscle at stage 21, but expression was stronger in the adult skeletal muscle preparation. As was the case with the other two K⁺ channel genes, XKv1.10 was also expressed in spinal cord neurons. The functional significance of this channel has yet to be determined, however given its abundance, we expect that this channel plays a major role in determining the electrophysiological properties of adult skeletal muscle.

The acquisition of the appropriate combinations of voltage gated ion channels in excitable cells during development determines their unique electrical properties. The results of this work show developmental changes in the

expression of three Kv1 K⁺ channel genes in muscle. Although the relative expression of the three transcripts was not carefully quantitated, the data suggest that XKv1.4 transcripts are more highly expressed in stage 21 muscle than XKv1.2' or XKv1.10 transcripts and that XKv1.10 transcripts are more highly expressed in adult muscle than XKv1.4 transcripts. No information is available on the electrophysiology of adult muscle in *Xenopus laevis* so the implications of the developmental expression of these K⁺ channels have yet to be determined. Developmental changes in K⁺ channel populations have also been reported for a number of systems including *Drosophila* muscle (Broadie and Bate, 1993b), rat myocardium, and *Xenopus* spinal cord (Ribera and Nguyen, 1993; Burger and Ribera, 1996; Gurantz *et al.*, 2000). Such changes in electrical properties of developing cells are strictly regulated and appear to be critical to normal development of electrical excitability and physiological function (Jones and Ribera, 1994).

Stage 14 is the earliest age from which muscle can be dissected from *Xenopus* embryos, however expression of muscle specific genes begins some time earlier. For example, expression of MyoD, a skeletal muscle specific transcription factor responsible for the terminal differentiation of skeletal muscle, begins during mesoderm induction at the time of gastrulation (stage 10) (Hopwood *et al.*, 1989; 1992). That XKv1.2' and XKv1.4 are expressed at such critical early times suggests that these channels may play important

developmental roles. The later appearance of XKv1.10 after muscle and neuron have differentiated suggests that it plays a different role.

The RT-PCR and RACE methods used to isolate K⁺ channel cDNAs in this study were specific to Kv1 channels. The results suggest that XKv1.2', XKv1.4, and XKv1.10 could represent the complete complement of Kv1 channels in *Xenopus* skeletal muscle. However, the results do not exclude the possibility that the screening technique failed to detect all possible Kv1 channels or channels from subfamilies Kv2, Kv3, and Kv4. Indeed, Kv2.2 (Burger and Ribera, 1996) probably makes a significant contribution to IK. The availability of the three cDNA sequences in the present study provides a mechanism of examining the contribution of each of these channels in the outward K⁺ current recorded from *Xenopus* muscle.

3.4.3 Implications for clustering

Previous work from our lab showed that inactivating K⁺ channels (producing I_{hK}) and voltage gated Na⁺ channels are co-clustered in high density at or near sites of nerve contact, where there is an accumulation of acetylcholine receptors. Similar observations of post-synaptic clustering of K⁺ channels mediating inactivating currents have been reported in the post-synaptic membrane of larval *Drosophila* neuromuscular junctions (Tejedor *et al.*, 1997); in rat supraoptic neurons (Alonso and Widmer, 1997); and somatodendritic compartments in granule cells of cerebellar cortex and CA1/CA3 pyramidal cells

of the hippocampus (Sheng *et al.*, 1992). The specific clustering of K⁺ channels at these locations is thought to involve interactions of the C-terminal T/SXV motif of the K⁺ channels with the PDZ domains of MAGUK proteins (Kim *et al.*, 1995; Kornau *et al.*, 1995). Interestingly, all three of the K⁺ channels isolated in the present study encode channel proteins with slightly different C-terminal T/SXV motifs. In addition, several proteins have been identified at the neuromuscular junction which contain PDZ domains including the MAGUK protein *Disks large* (Rafael *et al.*, 1998), α 1 and β 2-syntrophin (Peters *et al.*, 1997; 1998), and nNOS (Grozdanovic *et al.*, 1997; Abdelmoity *et al.*, 2000). Given the electrophysiological evidence for clustering K⁺ channels in *Xenopus* muscle and the unique C-terminal motifs of XKv1.2', XKv1.4 and XKv1.10 it is attractive to hypothesize that these K⁺ channels are specifically targeted to different locations in the muscle cell membrane via their different C-terminal T/SXV motifs.

Chapter 4

Discussion

4.1 Summary

Loose patch clamp studies have shown that K^+ channels do not show a specialized distribution with respect to the NMJ (Almers *et al.*, 1983; Roberts, 1987). However, re-examination of the ability of the loose patch clamp technique to accurately measure K^+ currents, and recent evidence of post-synaptic clustering of K^+ channels at central synapses (Alonso and Widmer, 1997) and invertebrate neuromuscular junctions (Tejedor *et al.*, 1997) suggested that there may indeed be post-synaptic clusters of K^+ channels in vertebrate muscle cell membrane. **Therefore, the hypothesis of the study described in Chapter 2 was that voltage gated K^+ channels are localized near synaptic sites in skeletal muscle.** To test this hypothesis, the spatial distribution of K^+ currents on the membrane of nerve contacted embryonic *Xenopus* myocytes in culture were examined using the macropatch variation of the patch clamp technique. The neuromuscular synapse of embryonic *Xenopus* muscle cells in culture was an ideal model system to carry out this investigation because of its simplicity and the detailed knowledge we have of its development. The findings of the macropatch study confirmed the hypothesis that K^+ channels are localized with synaptic sites in embryonic *Xenopus* muscle cells in culture. Specifically, the study described in Chapter 2 showed that two types of K^+ current (I_K and I_{IK}) and a Na^+ current (I_{Na}) were easily detected in myocytes using the macropatch technique and often more than 20 channels/ μm^2 were calculated to be present

in membrane patches. However, the membrane of the muscle cell in culture did not appear to have uniform distribution of these ion channels. Macropatches that contained I_{Na} almost always also contained I_{IK} , and the density of I_{IK} tended to be higher when co-localized with I_{Na} . The macropatches containing I_{IK} and I_{Na} tended to be most often found near sites of nerve contact and sites of AChR clustering. The macropatches containing I_K (and not I_{IK}) were also more often found near sites of nerve contact, however the distribution was not as dramatic as that of I_{Na} or I_{IK} . Also, in many macropatches no current was observable, and thus it appeared that a large proportion of membrane was barren of voltage gated K^+ and Na^+ channels.

In order for future experiments to further investigate (a) the spatial distribution of the K^+ channels in muscle *in vivo*, and (b) the functional role of the observed specialized distribution *in vivo*, it was necessary to determine the molecular identity of the K^+ channels which are expressed in *Xenopus* muscle. **Therefore the goals of the study described in Chapter 3 were to (a) isolate cDNAs encoding the K^+ channels which underlie the I_K and I_{IK} observed in *Xenopus* myocytes in culture (b) characterize the currents produced by the channels encoded by the isolated cDNAs and (c) determine the pattern of expression of the identified K^+ channels.** The first goal was achieved in that three Kv1 channel cDNAs were isolated from *Xenopus* muscle: XKv1.2', XKv1.4 and XKv1.10. The XKv1.2' and XKv1.4 cDNAs were highly similar to Kv1.2 and

Kv1.4 cDNAs isolated from numerous other species, but the XKv1.10 cDNA encoded a completely novel Kv1 isoform. The second goal was achieved in that preliminary voltage clamp data characterizing the currents produced by the XKv1.2', XKv1.4 and XKv1.10 channels expressed HEK 293 cells were obtained. XKv1.2' produced channels with non-inactivating kinetics similar to I_K , while XKv1.4 and XKv1.10 produced channels with rapidly inactivating kinetics similar to I_{K1} . However, characterization of the biophysical properties of the three K^+ channels was incomplete due to small sample size. The third goal of Chapter 3 was achieved in that XKv1.2', XKv1.4 and XKv1.10 mRNAs were shown to be differentially expressed in muscle cells and spinal cord neurons during development.

The macropatch study (Chapter 2) presents three novel findings relevant to the study of the development of the electrical properties of skeletal muscle. Firstly, it demonstrates that Na^+ channels cluster near synaptic sites in embryonic *Xenopus* muscle. Secondly, it demonstrates that K^+ channels have a specialized spatial distribution with respect to the synapse in the membrane of a vertebrate muscle cell, even though K^+ channels have been reported to show no such distribution (Almers *et al.*, 1983; Roberts, 1987). Thirdly, this study is the first to demonstrate co-clustering of Na^+ and K^+ channels. The first finding demonstrates similarity between development of the NMJ in *Xenopus* nerve-muscle co-culture and *in vivo* mammalian muscle, strengthening the validity of using *Xenopus* nerve-muscle co-culture as an *in vitro* model for studying the

development of the NMJ. The second and third findings demonstrate the possibility that the vertebrate NMJ is more complex than previously thought. This study also demonstrates that Na^+ channel clustering near the neuromuscular synapse does not depend on the development of deep post-synaptic folds.

The molecular biological studies described in Chapter 3 also make significant contributions to the study of the development of the electrical properties of *Xenopus* muscle. This study presents the first description of the Kv1.2' and Kv1.4 channel isoforms from *Xenopus*, and the first description from any organism of the novel XKv1.10 channel isoform. Furthermore the molecular biological studies describe the developmental expression of these genes in muscle. Together, the three channels are likely to be significant contributors to the K^+ current observed in *Xenopus* muscle. The electrophysiological properties of developing *Xenopus* muscle are known, and thus the present knowledge of the molecular identity of the underlying channels will allow investigation of the developmental and electrophysiological roles of I_K and I_{KX} .

Lastly, the cloning of new K^+ channel genes and functional expression of the channel proteins makes a significant contribution to the study of K^+ channel physiology. The observation that XKv1.2' and XKv1.4 produce K^+ currents similar to those produced by their mammalian orthologues demonstrates the conservation of structure and function of K^+ channels between mammals and amphibians. The observation that XKv1.10 produces an inactivating channel but

does not possess a "ball and chain" domain is especially exciting, given that most of the Kv α subunits cloned to date produce non-inactivating channels when expressed in the absence of β subunits. Dissection of the structure of XKv1.10 may lead to further understanding of the mechanisms and physiological roles of inactivating K⁺ currents in excitable cells.

4.2 Ionic currents recorded using the macropatch technique are indistinguishable from those recorded using other techniques

Using the macropatch technique, a voltage gated Na⁺ current, I_{Na} , and two outward voltage gated K⁺ currents, I_{K1} and I_{K2} , were observed in *Xenopus* myocytes grown in culture. The current/voltage relation of the Na⁺ currents as measured by the macropatch technique is indistinguishable from the same currents as measured using whole cell patch clamp (Kidokoro, 1992; Linsdell and Moody, 1995), and similar to the current ensembles reported by DeCino and Kidokoro, (1985). The kinetics and current/voltage relation of the K⁺ currents as measured by the macropatch technique are indistinguishable from the same currents as measured using whole cell patch clamp (Moody-Corbett and Gilbert, 1992a,b; Ribera and Spitzer, 1990; Currie and Moody, 1999; Kidokoro, 1992; Linsdell and Moody, 1995, Spruce and Moody, 1992) and single channel patch clamp techniques (Ernsberger and Spitzer, 1995). This confirms the validity of the macropatch technique in measuring voltage gated currents in

Xenopus muscle cells in culture, and the use of the macropatch technique as a tool to investigate the spatial distribution of K^+ channels in the cell membrane.

4.3 Macropatch recordings revealed that Na^+ and K^+ currents show specialized spatial distributions

The individual role of each of the K^+ current types has not been investigated. In part, the reason for this is that the two current types are neither pharmacologically nor physiologically separable (Moody-Corbett and Gilbert, 1992a,b). The macropatch recording technique allowed investigation of the spatial distribution of ion channels in the membrane of *Xenopus* muscle cells in culture, and these data can give some insight into the roles played by these ion channels.

4.3.1 Localization of I_{Na} near synaptic sites may facilitate initiation of action potentials in *Xenopus* myocytes

Embryonic *Xenopus* nerve-muscle co-cultures are commonly used in the study of synaptic development (Daggett *et al.*, 1996; Cohen, 1980; Wang *et al.*, 1995 for example), however the spatial distribution of Na^+ channels on nerve-contacted muscle cells in culture has not been previously investigated. Using the macropatch technique, the density of Na^+ channels in nerve contacted *Xenopus* myocytes was found to be higher at sites near sites of nerve contact

than at sites farther away. This pattern of spatial distribution is similar to the pattern observed in rat, chick, and adult frog muscle *in vivo*. The arrangement of Na^+ channels at the rat, chick, and adult frog muscle NMJ is thought to be essential in ensuring that a synaptic event triggers enough current to initiate an action potential in the muscle cell, thus leading to activation of the contractile machinery of the cell (Wood and Slater, 1997; Caldwell, 2000). Given that the action potential of embryonic *Xenopus* muscle cells is also Na^+ dependent (Decino and Kidokoro, 1986), the localization of Na^+ channels near the synapse in *Xenopus* muscle cells is also likely to ensure that a synaptic event triggers enough current to initiate an action potential.

The present novel observation that there is a high probability of finding voltage gated Na^+ channels at sites of nerve contact in one to three day old *Xenopus* muscle cells in culture is intriguing and important. The observation that Na^+ channels aggregate about these synapses, in manner which is similar to that observed in rodent and adult frog muscle *in vivo* (Caldwell *et al.*, 2000) and chick muscle *in vitro* (Angeleides, 1986) presents a model system to use in investigating the mechanisms that cluster Na^+ channels in cell membranes.

4.3.2 Localization of K^+ currents near synaptic sites may determine properties of electrical activity in *Xenopus* myocytes.

The hypothesis that K^+ channels are localized near synaptic sites in skeletal muscle was confirmed by the observations that the probability of observing I_K and I_{IK} was highest near sites of nerve contact, and also that I_{IK} was

co-localized with the Na^+ current and AChRs near sites of nerve contact. Clustering K^+ current at the neuromuscular synapse could have the effect of spatially co-localizing the repolarizing K^+ current with the depolarizing synaptic current and Na^+ current, and may thus have the functional consequence of enabling a rapid repolarization of the muscle cell after a depolarization. Additionally, the co-localization of a high density of K^+ current (i.e. I_{K}) with I_{Na} may ensure rapid removal of Na^+ channel inactivation, thus enabling the Na^+ channels to rapidly respond to a subsequent synaptic potential. Adding support to these notions is the observation that the channels which mediate I_{A} are found to be highly concentrated near the synaptic site in larval *Drosophila* muscle (Tejedor *et al.*, 1997). Such a localization may be essential to determine proper levels of electrical activity of the muscle which are required for normal development (Budnik *et al.*, 1990; Broadie and Bate, 1993; Jarecki and Keshishian, 1995).

The high density of I_{K} found at synaptic sites may attenuate activity caused by spontaneous release of ACh by motor neurons. Given that the threshold of activation of the I_{K} is only slightly positive to that of I_{Na} , and I_{K} does activate rapidly (Chapter 2, Moody-Corbett and Gilbert 1992a,b), it is possible that I_{K} may shunt depolarizing current through AChRs activated by spontaneous release of ACh from motoneurons. To fulfil such a role however, the clustering of channels producing I_{K} would have to be concurrent with the clustering of the AChRs. This remains to be examined.

The observation that I_{IK} , but not I_K , is co-clustered with Na^+ channels and AChRs near synaptic sites in *Xenopus* muscle is significant in that it suggests there is more than one structural type of channel. This observation is in agreement with Ernsberger and Spitzer (1995) who hypothesised the presence of multiple structurally different K^+ channels, based on the observed inactivation properties of single channel K^+ currents and the knowledge that K^+ channels are encoded by a large gene family. Moreover, in the present molecular biological studies three cDNAs encoding voltage gated K^+ channels were isolated from *Xenopus* muscle, demonstrating that at least three structurally different K^+ channels may underlie the observed K^+ currents. The presence of multiple structural K^+ channel subtypes with differential subcellular distributions suggests that the I_K and I_{IK} may subserve different electrophysiological roles, and may differentially contribute to determination of frequency, shape and duration of action potentials.

The observation that the density of I_{IK} is higher when co-localized with I_{Na} is consistent with an increase in channel density at these sites, not localized increases in channel conductances. The outward voltage gated K^+ channels expressed in *Xenopus* myocytes have been only observed to show single channel conductances ranging 17-23 pS (Ernsberger and Spitzer, 1995). However, it is possible that I_{IK} in *Xenopus* myocytes may be a Na^+ activated K^+ current (I_{KNa}). The apposition of K^+ channels and Na^+ channels exposes the K^+ channels to large and rapid local fluctuations in internal Na^+ concentrations,

which may in turn cause enhanced channel activation and thus alter the dynamics of muscle cell repolarization. The presence of I_{KNa} has been reported in a number of systems including invertebrate neurons (Hartung, 1985), chick ganglionic neurons (Bader *et al.*, 1985), chick brainstem neurons (Dryer *et al.*, 1989) and guinea pig myocytes (Kameyama *et al.*, 1984). The time course of inactivation and voltage of activation of I_{KNa} are also similar to I_{IK} . The role of such an I_{KNa} may be to further accelerate the repolarization phase of the action potential (Dryer *et al.*, 1989).

4.3.3 Localization of I_{IK} near AChRs may influence synaptic development by modulating tyrosine kinase signalling pathways.

The K^+ channels expressed in *Xenopus* myocytes, and their differential distribution may be involved in regulating intracellular tyrosine phosphorylation pathways, and thus development of the neuromuscular synapse. It has recently come to light that activity of Kv1 channels can differentially modulate activity of some tyrosine kinases (Holmes *et al.*, 1997). In particular, HEK 293 cells expressing the non-inactivating voltage activated mouse K^+ channels Kv1.3 and Kv1.5 showed a decrease in tyrosine phosphorylation of total endogenous cellular protein, as well as the tyrosine phosphorylation of heterologously expressed v-Src. Expression of mouse Kv1.4 (a channel which produces rapidly inactivating K^+ currents), and non-conducting mutants of mouse Kv1.3 caused no such effect, so the K^+ channel dependent decrease in levels of tyrosine phosphorylation appears to be correlated with the non-inactivating kinetics of

mKv1.3 and mKv1.5 channels. While it is possible that attenuation of tyrosine kinase activity is mediated by changes in internal K^+ concentration due to activity of transfected non-inactivating K^+ channels, the authors propose that attenuated tyrosine phosphorylation levels are coupled to the membrane potential, which is in part controlled by the K^+ channels. How the kinetics of the non-inactivating K^+ channels in HEK 293 cells mediate changes in tyrosine kinase activity is unknown. Importantly, co-expression of mKv1.3 with epidermal growth factor receptors (EGFRs) also caused a decrease in the tyrosine phosphorylation of the EGFRs with and without EGF treatment (Holmes *et al.*, 1997). As discussed below, tyrosine phosphorylation pathways that control development of the neuromuscular synapse within developing *Xenopus* skeletal muscle cells could be differentially modulated by means of the spatial distribution of the K^+ channels expressed.

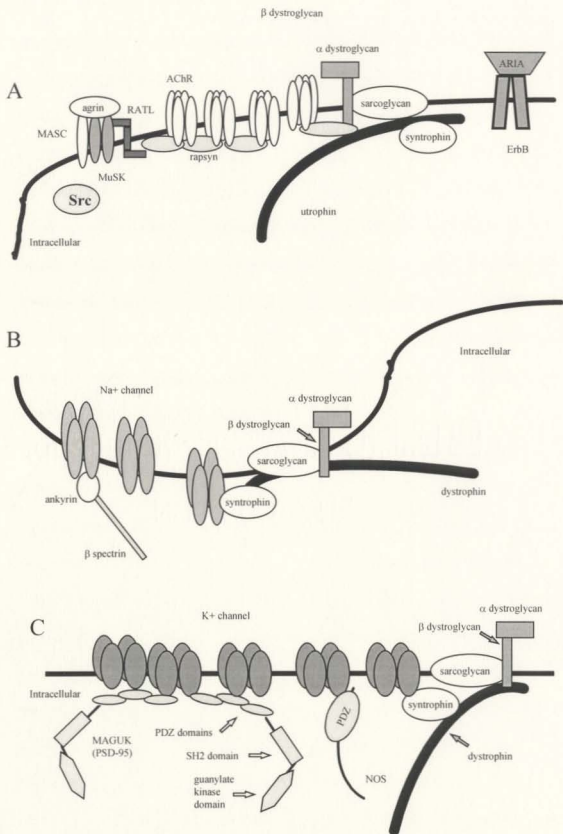
Initial development and maintenance of the aggregations of AChRs and the deeply invaginated membrane architecture (as found in mammals) is known to involve tyrosine kinase signalling (Wallace, 1994; Ferns *et al.*, 1996), however the intracellular signalling pathways are poorly understood. The current understanding posits that during development of the NMJ, motor neurons produce a signalling protein agrin, which binds with high affinity to a receptor in the muscle membrane that includes the receptor tyrosine kinase protein MuSK and an unidentified protein, MASC (muscle associated specificity component). A second unidentified transmembrane protein RATL (Rapsyn associated

transmembrane linker) then interacts with the agrin-bound receptor and rapsyn, a cytoskeletal protein which binds and clusters AChRs in the muscle post-synaptic membrane (McMahan, 1990; Glass *et al.*, 1996; Apel *et al.*, 1997, see also Figure 4.1A). A number of tyrosine kinase signalling events that seem to be involved in development of the NMJ have been recognized and are described below.

(1) Agrin activation of MuSK in myocytes is immediately followed by MuSK dimerization and tyrosine autophosphorylation (Hopf and Hoch, 1998; Apel *et al.*, 1997). This is followed by phosphorylation of tyrosine residues on the AChR β subunit (Hopf and Hoch, 1998; Apel *et al.*, 1997; Colledge and Froehner, 1998). Phosphorylation of AChR β subunits may occur via direct interaction with activated MuSK, or via binding of Src class protein tyrosine kinases (Fuhrer and Hall, 1996; Mohammed and Swope, 1999). Phosphorylation of AChR β subunits by the tyrosine kinase Src in quail fibroblasts resulted in binding of AChRs to elements of the cytoskeleton, indicating a role of tyrosine phosphorylation in anchoring (Mohammed and Swope, 1999). However the role of phosphorylation of the AChR β subunit is presently unclear, as mutational studies have shown that obliterating all of the potential tyrosine kinase sites in the β subunit does not prevent clustering by rapsyn in agrin stimulated myotubes (Meyer and Wallace, 1998).

(2) The AChR δ subunit is also a target of tyrosine phosphorylation.

Figure 4.1 Schematic diagrams showing proteins involved in anchoring ion channels at neuromuscular junctions. A. AChRs are anchored at the crests of post-synaptic folds of at the NMJ. Together with the myotube-associated component (MASC), MuSK binds agrin and becomes activated, and signals AChR aggregation through an unknown pathway that includes rapsyn and tyrosine phosphorylation. Activated MuSK also becomes part of the aggregation complex. The dystroglycan complex (including α and β spectrin, sarcoglycan and syntrophin) can also bind rapsyn and utrophin (the synaptic form of dystrophin). ARIA signalling via ErbB receptors and the non-receptor tyrosine kinase, Src, are also localized at the NMJ. B. Na^+ channels are anchored in the troughs of NMJ post synaptic folds. Clustering may be mediated by interactions with ankyrin and β spectrin and/or the dystroglycan complex via the PDZ domain of syntrophin. Neuregulin may be involved in local Na^+ channel synthesis. C. K^+ channels are anchored in the neuronal post synaptic membrane via interactions with PDZ domains of MAGUK proteins. Interactions with PDZ domains of syntrophin and nitric oxide synthase may also be possible. Note that the stoichiometry of the AChR and rapsyn interaction (1:1) is the only stoichiometry to have been determined; proteins are not drawn to scale. Models redrawn from (Hoch, 1999; Colledge and Froehner, 1998; Gee *et al.*, 1998; Tejedor *et al.*, 1997; LaRoche and Froehner, 1986; Hoch, 1999; Meier and Wallace, 1998).



Phosphorylation of AChR δ subunits by Src in quail fibroblasts also resulted in binding of AChR δ subunits to elements of the cytoskeleton (Mohammed and Swope, 1999). Furthermore, tyrosine phosphorylation of AChR δ subunits results in binding of Grb2, a modular adapter protein, and this interaction may be involved in further signalling processes utilizing the Grb2, son of sevenless (Sos), and Ras proteins (Colledge and Froehner, 1998). Grb2 acts as an adapter protein to bind a Ras guanine-nucleotide exchange factor, called Sos protein. Sos protein in turn regulates the action of Ras protein by promoting the exchange of GDP for GTP. Activation of the Ras/MAP kinase signal transduction pathway, can lead to increased AChR subunit gene expression (Colledge and Froehner, 1998, Tansey *et al.*, 1996).

(3) The neuregulin (also known as acetylcholine receptor inducing activity, or ARIA) signalling pathway is also localized at the developing NMJ in skeletal muscle. Neuregulin is an important factor in signalling transcription of NMJ specific genes such as utrophin, and AChR subunits from subsynaptic nuclei (Jo *et al.*, 1995; Sandrock *et al.*, 1997). Neuregulin binds to members of the EGF family of receptor tyrosine kinases (Altioik *et al.*, 1995), and activation of EGFRs results in activation of the phosphatidylinositol-3-kinase and MAP kinase pathways leading to activation of ets family transcription factors, binding to N-box promoters, and transcription of AChR subunits (Tansey *et al.*, 1996; Schaeffer *et al.*, 1998) as well as utrophin (Betz, 1998).

(4) A diffusible tyrosine phosphatase in muscle which is upregulated at

nerve contact has been implicated in contributing to the removal of extra-synaptic AChR hot spots, and maintaining the discreteness of synaptic clusters. The diffusible tyrosine phosphatase is proposed to create a steep gradient of tyrosine kinase signaling events beneath the nerve contact (Dai and Peng, 1998).

The co-localization a I_{K} (which may be produced by XKv1.4 or XKv1.10 channels, see sections 4.8, 4.9) with AChRs at synaptic sites is intriguing in light of the possibility that these currents, by their presence in the cell membrane may create a permissive microenvironment for the tyrosine kinase signalling events (1,2,3,4 above) which guide the development of the NMJ. The presence of I_K in the membrane may serve to contribute to the general attenuation the activity of tyrosine phosphorylation pathways throughout the nerve contacted muscle cells (Holmes *et al.*, 1997; Dai and Peng, 1998). However, the high density of I_K localized with AChRs near sites of nerve contact may create a local permissive state where the activity of tyrosine kinase pathways (1,2,3 above) is not inhibited. Such a permissive state might be essential for the differentiation and maintenance of a NMJ. Furthermore, perhaps modulation of the inactivation kinetics of I_K may serve to "fine tune" the activity of the tyrosine phosphorylation signalling pathways in the sub-synaptic domain (see section 4.9.2).

In summary, this section proposes that the spatial distribution of K^+ currents, especially I_K , in nerve contacted myocytes may allow tyrosine kinase activity, and may thus contribute to the development of the NMJ.

4.4 Macropatch recordings revealed that clustering of Na⁺ channels at the synapse occurs rapidly and does not require complex membrane infolding.

Clustering of Na⁺ channels near synaptic sites observed in Chapter 2 is similar to that observed in mammalian muscle *in vivo*. Two features of the observed clustering of Na⁺ channels however stand in contrast to observations from mammalian skeletal muscle *in vivo*. First, the clustering of the Na⁺ channels in *Xenopus* myocytes occurs over a very short time period as clusters were detectable as early as 24h after plating the cells, but Na⁺ channel clustering occurs more slowly in the rat. Lupa *et al.*, (1993) report that the appearance of a high density of Na⁺ channels at the rat muscle endplate is delayed compared to the AChR, with significant increases in Na⁺ channel density being first detectable five to ten days post-natal, and reaching adult levels sometime after 35 days post-natal. This may reflect a species difference in the rate of Na⁺ channel clustering.

The present study did not reveal the time course of ion channel clustering at sites of nerve contact, so the respective order of clustering of the AChRs, Na⁺ channel and K⁺ channels producing I_h near sites of nerve contact is unknown. Determination of the time course of Na⁺ and K⁺ channel clustering in *Xenopus* muscle cells in culture and *in vivo* will allow a more detailed comparison to the time course of rodent NMJ development (Lupa *et al.*, 1993).

The second difference between Na⁺ channel clustering in *Xenopus*

myocytes grown in culture and developing mammalian skeletal muscle *in vivo* is that while the membrane of *Xenopus* muscle in culture does not possess deep post-synaptic folds (Takahashi *et al.*, 1987) Na^+ channels still cluster near the synapse. This observation supports the view that the molecules involved in anchoring Na^+ channels in skeletal muscle membrane do so independently of the molecules which cause and maintain the deep synaptic folds observed in mammalian skeletal muscle (section 4.5).

4.5 Na^+ and K^+ channels may be co-clustered via interaction with PDZ domain containing proteins

The observation that Na^+ and K^+ channels are most often found near the synapse suggests that they are not freely mobile in muscle membrane, but are somehow anchored. This notion is supported by the observations that the Na^+ channels near the NMJ in innervated chick myocytes growing in culture are essentially immobile (Angelides, 1986) and that Na^+ and K^+ channels are similarly immobile in acutely dissociated adult frog skeletal muscle (Roberts *et al.*, 1986; Weiss *et al.*, 1986). The present observation that Na^+ and K^+ channels are not evenly distributed in the embryonic *Xenopus* muscle cell membrane begs the question of "How are these distributions maintained?".

4.5.1 Na⁺ channels may be clustered at the neuromuscular synapse by interactions with syntrophins, ankyrin, and spectrin

The mechanisms of anchoring Na⁺ channels in skeletal muscle cell membrane have been investigated. For example, mammalian skeletal muscle Na⁺ channel isoforms are known to possess C-terminal S/TVX motifs. This motif is thought to interact with the PDZ domain of the cytoskeletal associated protein syntrophin to achieve anchoring in the cell membrane (Gee *et al.*, 1998). In support of this notion, Na⁺ channels have been immunoprecipitated with the syntrophin/dystrophin complex from skeletal muscle (Gee *et al.*, 1998), and muscle specific Na⁺ channels have been shown to interact with α -1, β -1 and β -2 syntrophins *in vitro* (Gee *et al.*, 2000). Importantly, syntrophin has been shown to co-localize with synaptic and extra-synaptic AChR clusters in embryonic *Xenopus* skeletal muscle in culture (Dai and Peng, 1998). Furthermore, antibody staining shows co-localization of β spectrin and ankyrin with Na⁺ channels at the mammalian NMJ, suggesting that interactions between Na⁺ channels, β spectrin and ankyrin may also anchor Na⁺ channels at the NMJ (Flucher and Daniels, 1989; Wood and Slater, 1998). These two mechanisms may account for the observed Na⁺ channel clustering in *Xenopus* myocytes grown in culture (Figure 4.1B).

4.5.2 K⁺ channels may be clustered at the neuromuscular synapse by interactions with syntrophins, membrane associated guanylate kinases, and nNOS

As the present molecular biological studies have shown, there are at least three structurally different K⁺ channels (XKv1.2', XKv1.4, XKv1.10) expressed in *Xenopus* muscle cells. Importantly, these channels were shown to be members of the Kv1 subfamily, each possessing a variant of the S/TXV C-terminal motif. The C-terminal S/TXV motifs of the Kv1 channels have been shown to interact with PDZ domains of cytoskeletal membrane associated guanylate kinase (MAGUK) proteins such as PSD-95 (Kim *et al.*, 1995). For example, *Drosophila* Shaker K⁺ channels (equivalent to Kv1) are localized at the larval NMJ by interactions with the anchoring protein Dlg, the *Drosophila* orthologue of PSD-95 (Tejedor *et al.*, 1997). It is likely that the C-terminal motifs of XKv1.2', XKv1.4 and XKv1.10 can also interact with PDZ domain containing proteins (Figure 4.1C).

Additionally, the slight differences in the S/TXV C-terminal motifs of the XKv1.2', XKv1.4, XKv1.10 channels may account for the preferential localization of K⁺ channels near the synapse, and co-localization of channels that produce I_K with Na⁺ channels and AChRs. Firstly, it has been shown that each PDZ domain can have differential affinities for different ion channels. Heterologous expression of PSD-95 in HEK 293 cells effectively clustered heterologously expressed Kv1.4 K⁺ channels, but only infrequently did PSD-95 cluster

heterologously expressed Kv1.1 or Kv1.2 (Kim and Sheng, 1996). Also, PSD-95 has been shown to differentially cluster inward rectifier channels possessing variations of the C-terminal S/TXV/I motif (Nehring *et al.*, 2000). Furthermore, using filter overlay assays, Gee *et al.* (2000) have demonstrated that the syntrophins and MAGUK proteins have differential affinities for different ion channels including skeletal muscle Na⁺ channels, Kv1.4 and Kv1.5. Secondly, a number of PDZ domain containing proteins localize near the vertebrate NMJ: *Dlg* (Rafael *et al.*, 1998), α 1 and β 2-syntrophin (Peters *et al.*, 1998), and nNOS (Abdelmoity *et al.*, 2000; Grozdanovic *et al.*, 1997) have been shown to be expressed in rodent muscle and preferentially localized at the NMJ. Syntrophin has been localized to synaptic and extrasynaptic AChR clusters in embryonic *Xenopus* muscle in culture (Dai and Peng, 1998). Therefore, it is possible that a specific type of PDZ domain protein predominantly anchors each K⁺ channel. This specificity may determine the observed spatial distribution of K⁺ channels in muscle cell membrane.

4.5.3 Are channels mediating I_{Na} and I_K co-anchored via the same PDZ proteins?

Skeletal muscle Na⁺ channels and Kv1 subfamily K⁺ channels both possess variations of the C-terminal S/TXV/I motif. These motifs are bound by PDZ domains of a number of proteins which are found at muscle synaptic sites. Given the observation that 91.4% of the macropatches which contained Na⁺ currents also contained I_K, it is possible that the channels producing these

currents are co-anchored in the membrane via the same PDZ domain containing protein. In support of this, mouse Kv1.4 (which produces an inactivating K⁺ current) and skeletal muscle Na⁺ channels were all strongly bound by α -1 and β -2 syntrophin (Gee *et al.*, 2000). Further suggesting a common anchoring mechanism for Na⁺ and K⁺ channels, is the previous observation that Na⁺ and K⁺ channels are similarly immobile in the sarcolemma of frog skeletal muscle (Roberts *et al.*, 1986; Wiess *et al.*, 1986).

Determining whether PDZ domain containing proteins account for the observed differential distributions of I_K and I_{IK}, and the co-localization of I_{Na} and I_{IK} in *Xenopus* muscle will give further insights into the function of the distribution of these currents, as well as deepen our understanding of synaptic organization. An important step in understanding the mechanism and role of clustering of Na⁺ and K⁺ channels is essential to the elucidation of the molecular identities of these channels.

4.8 Three Kv1 channel cDNAs were isolated from *Xenopus* muscle

The observations made in Chapter 2 that different K⁺ current types were differentially distributed in the membrane of embryonic *Xenopus* muscle in culture, and the previous observation by Ernsberger and Spitzer (1995) that some K⁺ channels in the membrane of embryonic *Xenopus* muscle possessed convertible modes of inactivation suggested that the K⁺ current was produced by

a number of channel subtypes. Moreover, it seemed likely that one of the subtypes should possess structure that imparts rapidly inactivating kinetics. Therefore, one of the specific aims of this study was to isolate cDNAs encoding K⁺ channels responsible for the K⁺ currents observed in embryonic *Xenopus* muscle in culture. To this end, cDNAs encoding three putative K⁺ channels XKv1.2', XKv1.4 and XKv1.10, were isolated from *Xenopus* muscle. These Kv channels were identified as such by possession of a number of well conserved domains. These domains included a core of six hydrophobic membrane spanning α helices named S1-S6, and a pore domain named P1 (Heginbotham *et al.*, 1994). The S4 membrane spanning region, thought to be the voltage sensor, possesses seven positively charged residues at every third position (Bezanilla, 1991; Liman *et al.*, 1991; Stuhmer, 1991). Moreover, all three predicted proteins contained a T1 domain thought to be unique to Kv1 channels and thought to be responsible for multimerization of Kv1 subunits (Li *et al.*, 1992; Shen *et al.*, 1993). The XKv1.2' and XKv1.4 cDNAs were easily identified as K⁺ channels as they predicted proteins which were very similar to previously cloned Kv1.2 (Stuhmer *et al.*, 1989; Ribera, 1990) and Kv1.4 isoforms (Stuhmer *et al.*, 1989). The XKv1.10 cDNA however did not predict a protein that was a *Xenopus* orthologue of any known Kv1 channel isoform. However because XKv1.10 possessed significant similarity to other Kv1 channels though, it was deemed to be a new member of the Kv1 subfamily.

4.7 XKv1.2', XKv1.4 and XKv1.10 show unique patterns of expression during development

4.7.1 Expression of XKv1.2' is weak in embryonic skeletal muscle but strong in spinal cord

In addition to the isolation of three K⁺ channel cDNAs which are expressed in *Xenopus* muscle, another important finding of this study was the observation that these channels are differentially expressed during development of the embryo. Data in Figure 3.9A,B show that XKv1.2' expression initiated between stage 10.5 and stage 11.5, and increased through embryonic development. Although expression in embryonic muscle is detectable, expression is not detectable in adult muscle. Expression in stage 21 spinal cord however is very strong. Ribera (1990) reported that XKv1.2 expression was not detectable in muscle, but was detectable in the developing nervous system. Ribera also reported that XKv1.2 was a relatively rare transcript, making up about 0.001% of the total cellular mRNA at stage 18. It is likely that the detection of XKv1.2' in younger embryos and in muscle described in the present study was simply due to the superior sensitivity of RT-PCR compared to RNase protection assay used by Ribera (1990).

The pattern of expression of the Kv1.2' channel mRNA in *Xenopus* is similar to that observed in rat. Expression of XKv1.2 is weak in embryonic

Xenopus skeletal muscle, is not detectable in adult skeletal muscle and is strongly expressed in nervous tissue. Expression of rat Kv1.2 is also weak in developing skeletal muscle, undetectable in adult skeletal muscle and strong in the developing and mature nervous system (Lesage *et al.*, 1992). The apparent conservation of tissue specific and temporal expression of Kv1.2 in rat and frog suggests that this channel may play an important role in the development of skeletal muscle and neuron.

4.7.2 Expression of XKv1.4 is strong in skeletal muscle, and may be regulated by MyoD

Data in Figure 3.9A,B show that XKv1.4 expression in the whole embryo initiated between stage 8 and stage 10.5, and increased through development. It was expressed in the three stages of muscle tested, however expression at stage 21 appeared to be strongest.

Expression of XKv1.4 in *Xenopus* is also similar to Kv1.4 expression observed in rat (Lesage *et al.*, 1992). Both Kv1.4 channels are expressed in developing skeletal muscle, mature skeletal muscle and developing nervous system. Interestingly, three transcripts for rat Kv1.4 were detected but only one of which was detectable in muscle (Lesage *et al.*, 1992). Although the three transcripts for rat Kv1.4 were generated by alternative splicing in the 5' and 3' untranslated regions (Wymore *et al.*, 1996), alternative splicing in the 5' and 3' untranslated regions of the XKv1.4 transcript was not investigated. Such regulation of XKv1.4 may well occur in *Xenopus*.

The pattern of expression of XKv1.4 seems to parallel the expression of the muscle specific transcription factor MyoD. MyoD is activated immediately following mesodermal induction (gastrulation) in mesodermal tissue where it is thought to establish expression of muscle specific genes. Expression of MyoD becomes weaker in somites as the embryo matures (especially anterior somites), and expression is low in adult skeletal muscle (Hopwood *et al.*, 1989; 1992). This is significant in that the 5' UTR of the XKv1.4 cDNA includes five E-box sequences (MyoD-1 binding sites). These motifs might therefore drive strong expression of XKv1.4' during myogenesis (Puri and Sartorelli, 2000), and expression of XKv1.4 might be essential to the functional differentiation or maturation of skeletal muscle.

In contrast to XKv1.2', XKv1.4 was not strongly expressed in stage 21 spinal cord. A strong expression of XKv1.4 was not expected in stage 21 neurons because spinal cord neurons of this age do not possess an obvious inactivating current (termed I_{KA} by Ribera's and Spitzer's groups). The relative level of XKv1.4 expression is consistent with the observation that spinal cord neurons will develop a strong inactivating K^+ current at stage 26 (Desarmenien and Spitzer, 1993). If expression of XKv1.4 was examined in cDNA from spinal cord of stage 26 and older embryos, one might expect to see an increase in the amount of RT-PCR product.

4.7.3 Expression of XKv1.10 is strongest in adult skeletal muscle

Data in Figures 3.9 A,B show that XKv1.10 expression in the whole

embryo initiated between stage 17 and stage 21, and increased through development to the tadpole stage. XKv1.10 was expressed in stage 21 muscle, spinal cord and adult muscle, but not in stage 14 muscle.

The absence of expression of XKv1.10 in stage 14 skeletal muscle was interesting, as was the observation that XKv1.10 is more strongly expressed in adult muscle than embryonic muscle. This suggests that XKv1.10 may be an important channel for the expression of inactivating K^+ current in mature skeletal muscle, and that it plays a very different developmental role than either XKv1.2' or XKv1.4.

Consistent with its absence from stage 14 muscle but appearance in stage 21 muscle, the 5'UTR of XKv1.10 contained only one E-box motif, the binding site for the muscle specific transcription factor MyoD. Given that MyoD expression is weak in adult skeletal muscle (Hopwood *et al.*, 1989; 1992), the strong expression of XKv1.10 in adult skeletal muscle suggests that regulation of the XKv1.10 gene product requires other strong transcription factors.

Spinal cord neurons develop a strong inactivating K^+ current at stage 26 (Desarmenien and Spitzer, 1993). XKv1.10 expression is initiated by stage 21, and thus it is possible that this channel contributes to the inactivating K^+ current (I_{KA}). As with XKv1.4, determining if the expression of XKv1.10 increases in spinal cord at later times would provide clues to the importance of this channel in contributing to the inactivating current observed in neurons.

4.7.4 The developmental acquisition of ion channels in skeletal muscle appears to be tightly regulated

There is evidence suggesting that the XKv1.2, XKv1.4 and XKv1.10 channels contribute to the electrical properties of the developing muscle cells and neurons (see section 4.9), and it is significant that expression of each channel appears to have its own regulated pattern of expression as described in the sections above. K^+ channels are in a unique position to subtly modify electrical excitability, and therefore minute differences in the types of channels expressed in a cell, as well as spatial distribution can have important electrophysiological outcomes (Jan and Jan, 1997).

It is thought that patterns of ion channel expression are tightly regulated in developing *Xenopus* muscle. These cells show programmes of ion channel expression that determine patterns of electrical activity which appear to be essential for proper maturation. For example, the developmental acquisition of ion channels in embryonic muscle grown in culture is almost indistinguishable from the acquisition of ion channels by muscle which was induced *in vitro* by application of activin (Currie and Moody, 1999). Moreover, overexpression of voltage gated Na^+ channels in embryos caused a concomitant increase in the density of outward K^+ current in muscle cells in culture, and conversely, culturing cells in TTX caused a decrease in both Na^+ current and outward K^+ current (Linsdell and Moody, 1994; 1995). Interestingly, overexpression of an exogenous Kv1.1, an isoform normally found only in neural crest derivatives in

Xenopus (Ribera and Nguyen, 1993), accelerated expression of endogenous K⁺ channels (Spruce and Moody, 1995). These results strongly suggest that ion channel expression is tightly regulated during development and that perturbations in normal excitability may disrupt the normal maturation of excitable cells.

Currently, there is little idea of what roles are played by specific K⁺ channels during development and acquisition of electrical excitability. But now the roles played by the XKv1.2', XKv1.4 and XKv1.10 channels during development can be examined. The first step towards determining the contributions of each of these channels to development and function is to examine the properties of the K⁺ currents carried by each of the channels (section 4.8), and to then relate these currents to the currents observed in *Xenopus* myocytes in culture (section 4.9).

4.8 Properties of K⁺ currents produced by expression of XKv1.2', XKv1.4 and XKv1.10 in HEK 293 cells

4.8.1 Activation

The S4 regions of the predicted proteins for XKv1.2', XKv1.4 and XKv1.10 are identical. All three possess seven positively charged lysine or arginine at every third position in the manner of other Kv1 isoforms. Kv1 channels show

activation potentials between -60 mV and -20 mV (Conley, 1999). Therefore, functional XKv1.2', XKv1.4 and XKv1.10 channels were expected to show activation near these potentials. Although the data presented in Figures 3.6, 3.7 and 3.8 is preliminary, expression of XKv1.2', XKv1.4 and XKv1.10 cDNAs in HEK 293 cells clearly produced channels that activated within the expected range. XKv1.2' and XKv1.4 activated near -30 mV and XKv1.10 activated near -20 mV.

Early work examining the functional properties of XKv1.2 (Ribera, 1990; Ribera and Nguyen, 1993) reported that when overexpressed in *Xenopus* oocytes this channel activated at potentials positive to -20 mV. More recent work, however reported that the threshold of activation was between -40 and -30 (Lazaroff *et al.*, 1999). The difference probably lies in the recording technique used: the early work used two electrode voltage clamp, while the more recent work used the macropatch technique. The macropatch is a superior recording technique as large capacitive transients are eliminated, temporal resolution is improved and most importantly, applied voltages are more uniform across the fraction of *Xenopus* oocyte membrane tested (Lazaroff *et al.*, 1999; Baumgartner *et al.*, 1999). The reported threshold of activation for XKv1.2 (between -40 and -30, Lazaroff *et al.*, 1999) is similar to the thresholds observed for XKv1.2' in the present study.

The threshold of activation for the XKv1.4 channels observed in the present study was consistent with other observations which varied between -70

to -40 (Stuhmer, et al. 1989; Roeper *et al.*, 1997; Kupper, 1998; Petersen and Nerbonne, 1999; Jugloff *et al.*, 2000). Again, the differences may lie in the recording apparatus used as well as the expression system. In agreement with the present reported threshold of activation of XKv1.4, the threshold reported for activation of mammalian Kv1.4 in HEK 293 cells as recorded by whole cell patch clamp was between -40 and -30 (Jugloff *et al.*, 2000).

It is of considerable interest that Kv1.2' and XKv1.4 activated near -30 mV whereas XKv1.10 activated at about -20 mV even though all three channels possessed identical S4 domains. This observation strongly suggests that structures other than the amino acids of the S4 domain are involved in determining the voltage dependence of activation in voltage gated K⁺ channels. Although it is beyond the scope of this thesis to discuss the biophysical mechanisms determining voltage gating, there are some characteristics of the putative XKv1.10 channel protein which may lead to the depolarized threshold of activation. The putative amino acid sequences of XKv1.2' and XKv1.4 channels predict the presence of a site for N-linked glycosylation in the S1-S2 linkers, however this site is absent in the putative XKv1.10 channel. The presence of N-linked glycosylation, including sialic acid, has been shown to make a significant contribution to the voltage gating of Na⁺ channels (Bennett *et al.*, 1997). Specifically, reducing the sialylation on rat skeletal muscle Na⁺ channels caused a depolarized shift in the voltage of activation. The explanation was that removing glycosylation altered the local electric field sensed by the S4 activation

gate. Thus, the lack of N-linked glycosylation on XKv1.10 may have a similar effect on local electric fields and cause the observed depolarized shift of activation potential. It is also worth noting that there is a proline residue in the S3-S4 linker of the predicted XKv1.10 protein which is absent from XKv1.2' and XKv1.4 proteins. Proline is a rigid amino acid, and this may cause slight relative changes in the orientation, or angle of S4 in the membrane, and thus cause slight differences in voltage of activation. The significance of the depolarized voltage of activation of XKv1.10 with respect to the observed K⁺ currents in *Xenopus* myocytes will be discussed in a section 4.10.

4.8.2 Inactivation

4.8.2.1 XKv1.2' shows no inactivation

The currents recorded from HEK 293 cells transfected with XKv1.2' constructs activated rapidly and did not show significant inactivation over the 100 ms long depolarization pulses. This was expected as the putative XKv1.2' protein was essentially identical to XKv1.2 (Ribera, 1990) and rat Kv1.2 (Stuhmer *et al.*, 1989), which produce non-inactivating K⁺ channels.

4.8.2.2 XKv1.4 shows rapid N-type inactivation

The currents recorded from HEK 293 cells transfected with XKv1.4 constructs showed significant inactivation (49% inactivation, $\tau = 52$ ms) over the 100 ms long depolarization to 50 mV. The channels produced by XKv1.4 were predicted to produce a channel showing rapidly inactivating kinetics because the

putative XKv1.4 was very similar to mammalian Kv1.4 (Stuhmer *et al.*, 1989; Philipson *et al.*, 1990), with the major difference being in the chain portion of the ball and chain domain. When expressed in a heterologous expression system mammalian Kv1.4 cDNAs produce rapidly inactivating K⁺ channels (Stuhmer *et al.*, 1989; Lee *et al.*, 1996). During the process of "ball and chain" type inactivation or N-type inactivation the N-terminal portion of the K⁺ channel the first 20-36 amino acids of the channel form an inactivating particle which can swing up to block the pore and stop ion conduction (Hoshi *et al.*, 1990; Armstrong and Bezanilla, 1973; Tseng-Crank *et al.*, 1993). The reported rates of inactivation of mammalian Kv1.4 channels are similar to the rate reported here, but do show some variability which may be due to the expression system (Peterson and Nerbonne, 1999), oxidation of a cysteine residue (Ruppersberg *et al.*, 1991; Stephens *et al.*, 1996), or phosphorylation of the channel (Roeper *et al.*, 1997).

4.8.2.3 XKv1.10 shows rapid C-type inactivation

The currents recorded from HEK 293 cells transfected with XKv1.10 constructs activated rapidly and showed significant inactivation (22.5% inactivation, $\tau = 46$ ms over the 100 ms long depolarization to 50 mV). The observation that XKv1.10 encoded inactivating channels was interesting because the predicted amino acid sequence of XKv1.10 did not contain a "ball and chain" domain to confer rapid inactivation. Therefore, the inactivation must be of the rapid C-type (Hoshi *et al.*, 1991; Rasmusson *et al.*, 1998).

There are two types of C-type inactivation, slow and rapid. Despite being more potentially widespread than N-type inactivation, little is known about C-type inactivation. Past research has shown that even when the inactivating ball was deleted from *Shaker* channels, a slow inactivation process was still present (Hoshi *et al.*, 1990; Hoshi *et al.*, 1991). Mutations in amino acids in the S5, P and S6 domains near the C-terminal changed the slow inactivation rates in a number of cloned channels, and thus the slow inactivation was called C-type inactivation (Hoshi *et al.*, 1991). Recently, a Kv1.7 channel was cloned from mouse (Kalman *et al.*, 1998). mKv1.7 possessed no obvious structure to mimic the rapid "ball and chain" inactivation and yet when expressed in *Xenopus* oocytes, this channel produced K⁺ currents which showed rapid inactivation (τ was not reported, yet approximately 75% inactivation occurred over 50 ms; Figure 5, Kalman *et al.*, 1998). Until the present study, Kv1.7 has been the only Kv1 channel to show rapid inactivation that does not use the "ball and chain" mechanism.

The mechanism mediating C-type inactivation is poorly understood, however, there may be an interaction between amino acid residues around the pore which somehow squeeze the pore shut during sustained depolarization (Busch *et al.*, 1991). Furthermore, C-type inactivation probably requires cooperativity between the four subunits (Ogielska *et al.*, 1995; Panyi *et al.*, 1995). Comparison of XKv1.10 to other Kv1 channels did not reveal a greater similarity to Kv1.7, nor did comparison reveal a common unique structure or motif with

Kv1.7 which may be responsible for the observed rapid inactivation. Indeed, the properties of XKv1.10 and mKv1.7 inactivation seem to differ: XKv1.10 showed 22% inactivation with $\tau = 46$ ms, however the inactivation of Kv1.7 appeared to be more complete and much faster (Figure 5, Kalman *et al.*, 1998). C-type inactivation may be accomplished via different mechanisms in XKv1.10 and mKv1.7.

4.8.3 Sensitivity to TEA and 4-AP

4.8.3.1 XKv1.2' does not show sensitivity to TEA, but shows sensitivity to 4-AP

Unfortunately, XKv1.2' was the only channel of the three whose sensitivity to TEA and 4AP was investigated, and these are preliminary data. XKv1.2' showed insensitivity to external TEA, but showed sensitivity to 4-AP: 300 μ M produced between 30% to 100% block. These data are similar to those reported for XKv1.2, where XKv1.2 was insensitive to high concentrations of external TEA, but sensitive to 4-AP, showing 71% block at 1 mM (Ribera, 1990). Rat Kv1.2 showed similar sensitivity to these agents (Stuhmer *et al.*, 1989).

4.8.3.2 Mechanism of binding TEA and 4-AP

K⁺ channels vary greatly in their sensitivities to the blocking agents TEA and 4-AP. The external binding site for TEA is an aromatic amino acid in the X position downstream of the signature sequence TVGYGDMX (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992; Kavanaugh *et al.*, 1992;

Gomez-Hernandez *et al.*, 1997). XKv1.2', XKv1.4 and XKv1.10 all have non-aromatic amino acids in this position, and were thus predicted to be insensitive to external TEA. This hypothesis was confirmed for only XKv1.2'.

4-AP is membrane permeant, and is thought to bind to cytoplasmic parts of S5 and S6 which line the inner mouth of the pore during the process of activation to block conduction through the pore (Kirsch *et al.*, 1993; Shieh and Kirsch, 1994; Zhang *et al.*, 1999). Key residues are thought to be a leucine in the S5, a threonine, and two valines in the S6 (Kirsch *et al.*, 1993; Shieh and Kirsch, 1994; Zhang *et al.*, 1999). Even though mammalian Kv1.2 and Kv1.4 possess the key threonine and valines of the S6, Kv1.2 is reported to be ten times more sensitive to 4AP than Kv1.4 (Stuhmer *et al.*, 1989). Accessibility of the binding sites during activation is thought to underlie the sensitivity of Kv1.2. XKv1.2', XKv1.4 and XKv1.10 also possess the key threonine and valines of the S6, however they do not possess the leucine of the S5. Based on the sensitivity of the mammalian orthologues, it was predicted that XKv1.2' would be sensitive to block by 4-AP, but XKv1.4 would not be. Sensitivity of XKv1.10 was not predicted. The predicted sensitivity of XKv1.2' to 4-AP was confirmed.

4.9 Evidence suggesting that XKv1.2', XKv1.4 and XKv1.10 channels underlie the K⁺ currents observed in *Xenopus* myocytes in culture, and in neurons *in vivo* and in culture

4.9.1 XKv1.2' appears to contribute to I_K

XKv1.2' is likely to contribute to I_K observed in *Xenopus* myocytes in culture. We have presently demonstrated that the XKv1.2' cDNA produces a non-inactivating current with a threshold of activation similar to that of I_K in myocytes (Chapter 2). XKv1.2' is also expressed in *Xenopus* skeletal muscle at times when I_K is detectable by whole cell patch clamp (Moody-Corbett and Gilbert, 1992a,b; Linsdell and Moody, 1995) and single channel patch clamp (Ernsberger and Spitzer, 1995).

The present evidence suggests that XKv1.2' contributes to I_K , however the data do not rule out the possibility that other K^+ channels may also make a significant contribution. A likely candidate to contribute to skeletal muscle I_K is the XKv2.2 channel (Burger and Ribera, 1996), which is expressed in *Xenopus* embryonic skeletal muscle starting at stage 22.

XKv1.2' also appears to make a contribution to the non-inactivating K^+ current observed in embryonic *Xenopus* spinal cord neurons *in vivo* and in culture. XKv1.2' is expressed as early as stage 11.5, and I_K currents are detectable in spinal cord neurons a few hours later (Spitzer and Lamborghini, 1976; Baccaglioni and Spitzer, 1977; and O'Dowd *et al.*, 1988). Thus, development of I_K which is thought to be crucial to the maturation of the action potential (Ribera, 1990; Barish, 1986; O'Dowd *et al.*, 1988) and morphological differentiation of neurons (Jones and Ribera, 1994) may be encoded by XKv1.2'.

4.9.2 XKv1.4 appears to contribute to I_{KX}

Several lines of evidence suggest that XKv1.4 contributes to I_{KX} in *Xenopus* myocytes. XKv1.4 encodes an inactivating current, and XKv1.4 is expressed in myocytes from the earliest times when I_{KX} is observed (Spruce and Moody, 1992; Moody-Corbett and Gilbert, 1992a,b; Ernsberger and Spitzer, 1995). Also, XKv1.4 has an inactivation time constant ($52.1 \text{ ms} \pm 6.0$, $n = 3$) which is similar to one of the time constants ($72.2 \text{ ms} \pm 30\text{ms}$) reported for the inactivation of I_{KX} in whole cell current recordings (Moody-Corbett and Gilbert, 1992a;). Lastly, the inactivation rate of the XKv1.4 channel is expected to be sensitive to oxidation due to the presence of a cysteine residue in either the ball domain (Ruppersberg *et al.*, 1991) or the S6 region (Stephens *et al.*, 1996). This property is consistent with sensitivity of the inactivating current of *Xenopus* myocytes to oxidative and reductive conditions as described by Ernsberger and Spitzer (1995).

Another line of evidence suggests that XKv1.4 contributes to I_{KX} . Whole cell recording shows two components of I_{KX} inactivation: a slow component ($\tau = 70 \text{ ms}$), and a fast component ($\tau = 4 \text{ ms}$) (Moody-Corbett and Gilbert, 1992a). Two properties of mammalian Kv1.4 have been demonstrated to allow switching of inactivation from slow to rapid, and the structural elements which determine these properties are conserved in XKv1.4. Firstly, oxidation and reduction of cysteine residues of Kv1.4 have been shown to switch inactivation of Kv1.4: inactivation was rapid in the reduced state and very slow or almost non-existent

in the oxidized state (Ruppersberg *et al.*, 1991; Stephens *et al.*, 1996). XKv1.4 possesses the cysteines which render mouse Kv1.4 sensitive to oxidation and reduction. A combination of channels in the oxidized state and reduced state may lead to a K^+ current with two components of inactivation. Secondly, the presence of the fast and slow components of inactivation may be due to phosphorylation of XKv1.4. Specifically, phosphorylation by CaMKII at a site near the N-terminus can lead to drastic changes the rate of inactivation of Kv1.4 (Roeper *et al.*, 1997). The unphosphorylated channel showed a $\tau = 4.2$ ms, while the phosphorylated channel showed a $\tau = 40$ ms or greater. XKv1.4 possesses a putative N-terminal CaMKII phosphorylation site. Therefore, a combination of channels in the CaMKII unphosphorylated state and phosphorylated state may lead to a whole cell current with two components of inactivation. In summary, modification of XKv1.4 by either cysteine oxidation/reduction and state of phosphorylation by CaMKII in *Xenopus* myocytes in culture can potentially explain the two components of inactivation observed in I_{K1} recorded from *Xenopus* myocytes in culture. It is not presently known whether or not the effects of cysteine oxidation/reduction and state of phosphorylation by CaMKII are additive, but this may explain the variability observed in the inactivation rate of I_{K1} in *Xenopus* muscle cells in culture (Chapter 2; Moody-Corbett and Gilbert, 1992b).

Similarities between the inactivation properties of XKv1.4 and I_{K1} suggest that XKv1.4 may also underlie, or contribute to I_{K1} observed in *Xenopus* spinal

cord neurons. Firstly, the rates of inactivation of XKv1.4 and I_{KA} are similar, with the inactivation time constant of I_{KA} being reported to be on the order of 20 ms. A property of the I_{KA} in spinal cord neurons is its sensitivity to inactivation by depolarizing prepulses (Ribera and Spitzer, 1990). The V_{50} (potential of half inactivation) for steady state inactivation of I_{KA} was measured to be near -40 mV *in vivo* (Desarmenien and Spitzer, 1993) and in culture (Ribera and Spitzer, 1990). rKv1.4 is sensitive to depolarizing prepulses, showing V_{50} values of -37.8 mV to -44.5 mV, depending on phosphorylation state (Roeper *et al.*, 1997). Because of its similarity with rKv1.4, XKv1.4 is also predicted to show V_{50} values near -40 mV, supporting the hypothesis that XKv1.4 may contribute to I_{KA} .

4.9.3 XKv1.10 appears to contribute to I_{IK}

XKv1.10 may also contribute to I_{IK} observed in *Xenopus* myocytes in culture. XKv1.10 encodes an inactivating current, XKv1.10 is expressed in myocytes at stage 21, and I_{IK} is known to be expressed in myocytes in culture a few days after this time (Moody-Corbett and Gilbert, 1992a,b; Linsdell and Moody., 1992). Also, XKv1.10 has an inactivation time constant ($\tau = 46.3 \text{ ms} \pm 4.9$) which is similar to one of the time constants ($72.2 \text{ ms} \pm 30\text{ms}$) reported for the inactivation of I_{IK} in whole cell current recordings (Moody-Corbett and Gilbert, 1992a;).

Based on voltage of activation, it is also possible that XKv1.10 contributes to the delayed increase in I_{IK} observed in spinal cord neurons (O'Dowd *et al.*, 1988; Desarmenien and Spitzer, 1993). The voltage of activation of XKv1.10 (about -20 mV) is similar to the voltage of activation observed in spinal cord

neurons (O'Dowd *et al.*, 1988; Desarmenien and Spitzer, 1993; Ribera and Spitzer, 1990), however the steady state inactivation properties of XKv1.10 have not yet been investigated.

4.10 Roles of XKv1.2', XKv1.4 and XKv1.10 in skeletal muscle

Knowledge of the identities of some of the K^+ channels which appear to underlie I_K and I_{IK} in *Xenopus* myocytes in culture and spinal cord neurons can lead to generating hypotheses about the developmental roles played by the channels and the currents they generate.

XKv1.2' is weakly expressed in developing muscle as soon as it is morphologically identifiable, and in muscle during the period when motor neurons are inducing formation of the NMJ (Blackshaw and Warner, 1976; Kullberg *et al.*, 1977; Chow and Cohen, 1978). The XKv1.2' channel produces a non-inactivating K^+ current when expressed in heterologous cells, and is therefore likely to produce non-inactivating channels when expressed *in vivo*. The channel is expressed before the appearance of I_{Na} , and therefore is likely to act stabilizing membrane potential in very young (stage 14) muscle. The early acquisition of K^+ channels may well serve to prohibit long membrane depolarizations from AChRs and Na^+ channels. XKv1.2' activates at potentials slightly positive to the threshold for activation I_{Na} , and would therefore be expected to be involved in the repolarization of muscle after activation of I_{Na} . I_K is not as highly localized near

nerve contact as I_{KX} , nor is I_K co-localized with AChRs and I_{Na} , thus XKv1.2' may participate in selectively repolarizing extra-synaptic membrane. The physiological purpose for "selection" of XKv1.2' over other Kv channels for such a purpose remains unknown. Perhaps Kv1.2' has significant properties that remain to be elucidated. Conversely, perhaps XKv1.2' is a generic non-inactivating K^+ channel expressed at early times in the development of muscle because this channel has the proper regulatory features, and any Kv channel which produces non-inactivating current would equally suffice for the purpose.

XKv1.4 is weakly expressed in developing muscle as soon as muscle is morphologically identifiable, but in contrast to XKv1.2', becomes more strongly expressed as the embryonic muscle develops functional synapses. The XKv1.4 channel produces an inactivating K^+ current when expressed in heterologous cells, and is likely to produce inactivating channels when expressed *in vivo*. Like XKv1.2', XKv1.4 is expressed before the appearance of I_{Na} , so it may also act in stabilizing membrane potential in very young (stage 14) muscle, and repolarizing the membrane during an action potential. In culture, I_{KX} accumulates near synaptic sites with AChRs and Na^+ channels. Given that the threshold of activation of XKv1.4 is only slightly positive to that of I_{Na} , XKv1.4 may also be involved in limiting action potentials by shunting depolarizing current.

The presence of a high density of non-inactivating K^+ current is known to inhibit tyrosine phosphorylation of Src kinase and EGF receptors (Holmes *et al.*, 1997), which are thought to be involved in NMJ tyrosine phosphorylation

signalling pathways (Colledge and Froehner, 1997). In light of this, in a previous section (4.3.3), it was proposed that localization of I_{KX} at the developing synaptic site in muscle cells in culture may create a sub-synaptic microenvironment which is permissive to the tyrosine kinase pathways signalling the formation of AChR aggregates. Further to this hypothesis, it is possible that the modulation of the kinetics of I_{KX} (presently proposed to be produced by XKv1.4) by cysteine oxidation (Stephens *et al.*, 1996; Ruppertsberg *et al.*, 1991) and phosphorylation by CaMKII (Roeper *et al.*, 1997) may influence the permissiveness of the sub-synaptic microenvironment to tyrosine kinases and play an important role in aggregation of AChRs and the development of the NMJ. This hypothesis remains to be tested, however knowing the genetic sequence of the K^+ channel producing an inactivating K^+ current provides an excellent tool to carry out the necessary experiments.

XKv1.10 transcripts are absent from skeletal muscle at very early times (stage 14), but expression is commenced later near the time motor neurons induce formation of the NMJ (Blackshaw and Warner, 1976; Kullberg *et al.*, 1977; Chow and Cohen, 1978) and I_{KX} is expressed (Linsdell and Moody, 1994; 1995). Expression of XKv1.10 appears to be strongest in adult skeletal muscle. These observations preclude the possibility that XKv1.10 is necessary for the early maintenance of muscle membrane potential, or differentiation of muscle, and make the possibility unlikely that XKv1.10 is involved with aggregation of AChRs at the synapse. Furthermore, given that XKv1.10 produces an inactivating K^+

current, but shows threshold of activation which is depolarized compared to that of the observed I_{K1} (Chapter 2; Moody-Corbett and Gilbert, 1992a,b), it is unlikely that this channel comprises the dominant proportion of the channels which produce I_{K1} near sites of nerve contact in myocytes in culture.

The pattern of expression of the XKv1.10 transcript and the depolarized threshold of activation of the XKv1.10 channel suggest that this channel plays a role in permitting a high rate of action potential firing in mature muscle. XKv1.10 activates some 20 mV positive to the threshold of activation of Na^+ channels. Such a channel could not function to shunt depolarizing current and attenuate activity as does the neuronal I_A . (Daut, 1973; Cassell and McLachlan, 1986; Segal *et al.*, 1984; Hille, 1992). Instead, this channel would allow depolarizing currents to rapidly bring the post-synaptic membrane to threshold, and function only in the repolarization phase of an action potential. The electrophysiological properties of adult *Xenopus* skeletal muscle are as yet unknown, however such a K^+ channel may have significant importance in skeletal muscle as it would limit action potential duration without affecting the safety factor of action potential initiation.

It is worthwhile to note however that while expression of XKv1.2', XKv1.4 and XKv1.10 was detected in developing *Xenopus* muscle and spinal cord neurons, it is possible that not all cells will express these channels. For example, whole cell patch clamp recordings have shown that 7-9 hour old aneural embryonic *Xenopus* myocyte cultures predominantly express either I_K or I_{K1}

(Moody-Corbett and Gilbert, 1992b). Moreover, in spite of the observation that the non-inactivating voltage activated K^+ current develops in all *Xenopus* spinal cord neurons in a fairly homogeneous manner, the spectrum of ion channels that underlie this current differs between subsets of neurons (Vincent *et al.*, 2000; Gurantz *et al.*, 1996). The significance of the observed heterogeneity is not yet understood.

4.11 Future directions

The studies described in Chapter 2 demonstrate that K^+ channels which produce I_{K1} in *Xenopus* myocytes are preferentially localized with Na^+ channels near sites of nerve contact. Having identified three K^+ channels that are expressed in myocytes at this age (Chapter 3), there are clear studies to pursue.

4.11.1 Determining the role of XKv1.2', XKv1.4 and XKv1.10 in electrical excitability.

One study to pursue is to fully characterize the K^+ channels encoded by the XKv1.2', XKv1.4 and XKv1.10 cDNAs. Examining the kinetics and pharmacology of the currents produced by expression of the K^+ channel clones in HEK cells was initiated in the present study, but this task remains incomplete. Following characterization of the biophysical properties of the XKv1.2', XKv1.4 and XKv1.10 channels, the effect of I_K and I_{K1} on the properties of an action potential should be determined. Co-expression of a voltage gated Na^+ channel

with either XKv1.2', XKv1.4 or XKv1.10 in a heterologous system such as HEK 293 cells ought to permit an action potential in those cells, as they would have the necessary basic ionic channels. Comparing the shape, duration, and frequency of action potentials in cells expressing with Na⁺ channels and K⁺ channels that produce either I_K, I_{KX} or both K⁺ currents would lead to the determination of the role of each of those currents during an action potential. Such an analysis has not to my knowledge been previously performed, and the determination of possible differential roles of Kv1.2, Kv1.4 and Kv1.10 isoforms during an action potential could contribute to a further understanding of how K⁺ channels regulate excitability of muscle cells in culture, and perhaps also neurons *in vivo*.

4.11.2 Determining the spatial distribution of XKv1.2', XKv1.4 and XKv1.10, and the mechanisms that anchor these K⁺ channels

Another important series of experiments that ought to follow from the present studies is to visually identify the spatial expression of the XKv1.2', XKv1.4 and XKv1.10 channels as this will confirm and expand the data presented in Chapter 2 and Chapter 3. One approach is to use immunocytochemistry to directly label the ion channels. This is a straightforward exercise with the XKv1.2 channel, as antibodies against the mammalian protein are commercially available, and the epitope used to make the mammalian Kv1.2 antibodies is identical to that of XKv1.2'. Commercial antibodies against mammalian Kv1.4 however are designed against an epitope in the N-terminal region which does not

show complete similarity to XKv1.4, and thus these antibodies may or may not bind to XKv1.4. Since XKv1.10 is a novel protein, there is no commercial antibody available. In order to carry out XKv1.10 immunohistochemistry, an antibody will have to be made. These data would be expressed in terms relative to nerve contact and induction of AChR clustering. These experiments are important in that they will identify the precise spatiotemporal distribution of three K^+ channels whose regulation may be critical in the development of the NMJ.

The hypothesis that differential subcellular distribution of K^+ channels is achieved by differential associations of PDZ domain containing proteins with the different C-terminal S/TXV motifs found on the XKv1.2', XKv1.4 and XKv1.10 channels should be tested. Expression constructs encoding green fluorescent protein fused to the N-terminal of the K^+ channel proteins (as opposed to the C-terminal constructs engineered by Kupper, 1998), could be engineered and transfected into *Xenopus* muscle cells in culture. Preliminary experiments transfecting *Xenopus* GFP expression vectors (kindly provided by Dave Turner and Ralph Rupp) alone suggest that transfection of *Xenopus* muscle cells in culture is a viable experiment. The membrane targeting of each fluorescent-tagged channel could be visualized using confocal microscopy. These experiments would demonstrate the specific membrane targeting of the three channel proteins, and would suggest interaction of the free C-terminal T/SXV domain with PDZ domain containing proteins. Specific experiments to determine which (if any) PDZ proteins interact with K^+ channels would follow. Describing

the molecular interactions that determine structure and function of the NMJ is important in its own right, and may prove to be useful in understanding the physiology central synapses. Furthermore, understanding spatial distributions of ion channels and the mechanisms which anchor ion channels in cell membranes would have a broad significance in gaining a greater understanding of the physiological functioning of ion channels.

4.11.3 Determining the role of I_{K} in tyrosine phosphorylation and development of the NMJ

Tyrosine kinase signalling is recognized to be important in the development of the NMJ, as well as numerous other cellular processes including proliferation, differentiation and survival. Evidence presented in section 4.3.3 suggests that the co-localization of I_{K} with AChRs may contribute to the development of the NMJ. Therefore it is important to test the hypothesis that expression of XKv1.2' will decrease levels of tyrosine phosphorylation in muscle cells while expression of rapidly inactivating XKv1.4 will not. A first step to testing this hypothesis is to transfect muscle cells in culture with K^{+} channel cDNA constructs (sense and antisense) and examine levels of protein tyrosine phosphorylation at a later point as described in Holmes *et al.*, (1997) for HEK 293 cells. Another important preliminary experiment to test the hypothesis that K^{+} channels play a role in development of the NMJ is to transfect myocytes in culture with sense and antisense K^{+} channel cDNA constructs prior to nerve contact and examine patterns of tetramethylrhodamine-labeled α -bungarotoxin

staining. This will determine if spontaneous AChR clustering and nerve induced AChR clustering have been altered. Understanding the roles played by K^+ channels in tyrosine kinase signalling and development of the NMJ may lead to a greater understanding of the mechanisms that regulate development and plasticity of central synapses.

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